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00550376004
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4. Title of the invention

CORE 2 GLcNAc-T INHIBITOR

5. Name of your agent *(if you have one)*

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Core 2 GlcNAc-T Inhibitor

Field of the Invention

The present invention relates to a composition obtainable from fenugreek seeds and comprising a core 2 GlcNAc-T inhibitor, a method of preparing the composition and a core 2 GlcNAc-T inhibitor isolatable from fenugreek seeds. The present invention further provides use of the composition or the core 2 GlcNAc-T inhibitor for the manufacture of a medicament for the treatment or prevention of a disease associated with raised activity of core 2 GlcNAc-T, in particular inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

It has been demonstrated in the present invention that a composition prepared from fenugreek seeds can inhibit glucose-induced activity of core 2 GlcNAc-T and glucose-induced binding of human leukocytes to cultured bovine retinal capillary endothelial cells. The administration of the inhibitor to patients can prevent or treat the abnormal formation of core 2 O-glycans and sialyl Lewis^x (sLe^x) by directly inhibiting raised activity of core 2 GlcNAc-T in disease states, such as inflammation, diabetic cardiomyopathy, cancer and diabetic retinopathy.

Background

Membrane associated carbohydrates are mainly in the form of oligosaccharides covalently attached to proteins forming glycoproteins. The predominant sugars found in glycoproteins are glucose, galactose, mannose, fucose, GalNAc, GlcNAc and NANA. The distinction between proteoglycans and glycoproteins resides in the level and types of carbohydrate modification. The carbohydrate modifications found in glycoproteins are rarely complex: carbohydrates are linked to the protein component through either O-glycosidic or N-glycosidic bonds. In O-linked glycoproteins, the carbohydrate directly attached to the protein is GalNAc; in N-linked glycoproteins, it is GlcNAc.

O-glycosylation involves an initiation step in the Golgi, followed by a processing step. The initiation step O-glycosylation is the addition of GalNAc to serine (or threonine) of a glycoprotein. This initiation step is complex and is carried out by a large family of homologous UDP-GalNAc:polypeptide GalNAc-transferases. The polypeptide GalNac-transferase isoforms have distinct enzymatic properties and are differentially expressed, thus presumably allowing for a high level of control in determining sites of O-glycan attachment in proteins. Many of these enzymes are exquisitely sensitive to other events taking place within the cell in which the glycoprotein is expressed. The populations of sugars attached to an individual protein will therefore depend on the cell type in which the glycoprotein is expressed and on the physiological status of the cell, and may be developmentally and disease regulated.

The processing step involves elongation, branching and terminal modification of the O-glycans. Essential steps in O-glycan elongation and branching are catalysed by multiple glycosyltransferases isoforms from families of homologous glycosyltransferases. Depending on which saccharide groups are subsequently attached to this first GalNAc residue, O-glycans are divided into four major subtypes (Figure 1). The **core 1** structure is

formed by addition of galactose to form Galβ1-3GalNAc-αSer/Thr. The **core 2** structure requires the core 1 structure as substrate and is formed by addition of GlcNAc to form Galβ1-3(GlcNAcβ1-6)GalNAc-αSer/Thr. The **core 3** structure is formed by the addition of GlcNAc to form GlcNAcβ1-3GalNAc-αSer/Thr. The **core 4** structure requires the core 3 structure as substrate and is formed by addition of GlcNAc to form GlcNAcβ1-3(GlcNAcβ1-6)GalNAc-αSer/Thr. Other modifications to the core GalNAc structure have also been found, but appear to be uncommon. All these core structures are further modified by galactosylation, sialylation, fucosylation, sulfation or elongation.

Cell surface carbohydrates (O-glycans) are known to play a crucial role in mediating cell-cell interactions in development and certain disease states. The developmentally-regulated patterns of glycoprotein glycosylation are determined largely by the activity and specificity of glycotransferase enzymes, such as UDP-GlcNAc:Galβ1-3GalNAcαRβ1-6-N-acetylglucosaminyltransferase (core 2 GlcNAc-T) expressed in the Golgi (1-2). Core 2 GlcNAc-T plays a crucial role in the biosynthesis of O-linked glycans by converting core 1 to core 2 structures (3-4) and represents an important regulatory step for the extension of O-linked sugars with polylactosamine (i.e. repeating Galβ1-4GlcNAcβ1-3), a structure associated with malignant transformation (5-6).

GlcNAc-T activity appears to be regulated by factors that have an impact on intracellular signalling and development status of the cell. Changes in the activity of core 2 GlcNAc-T have been associated with various disease states, such as T-cell activation, cancer, myeloblastic leukaemia, myocardial dysfunction and inflammation (7-18). Regulation of core 2 GlcNAc-T is thought to be important, because addition of lactosamine structures and subsequent modification with fucose and sialic acid results in the formation of Lewis^{x} and sialyl-sialyl Lewis^{a}, and Lewis^{x} sugar groups that constitute ligands of selectins.

Core 2 GlcNAc-T and inflammation

Inflammation is how the body generally responds to infection or to some other form of trauma. One of the major events during inflammation is the movement of cells of the immune system from the blood stream to the infected or injured area. Once at the site of injury, these cells are responsible for the isolation, destruction and removal of the offending agent (regardless of whether it is an infectious agent, foreign substance or necrotic tissue). Chemicals known as cytokines and prostaglandins control this process and are released in an ordered and self-limiting cascade.

Acute inflammation, characterised by short duration (minutes to days), is essential for health, but sometimes the inflammatory process does not end when appropriate, and it is this that causes problems. Chronic inflammation is characterised by long duration (days, weeks, months and even years), lymphocytes and macrophages, tissue destruction and repair, and vascular proliferation and fibrosis. Inflammation can also be triggered inappropriately by the body's normal constituents and plays a role in common diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease.

Many cell adhesion molecules are known to be involved in the process of inflammation. At the site of inflammation, leukocytes first adhere to the vascular endothelial cells prior to the extravasation process. It is postulated that the members of a cell adhesion family, selectins, play a crucial role in the initial adhesion of leukocytes to endothelial cells, while

other adhesion molecules such as integrins and members of the Ig superfamily are involved in a later process. Cell adhesion mediated by selectins and their carbohydrate ligands arises to the tethering and rolling of leukocytes on endothelial linings. This leads to the secondary firm adhesion and signal transduction mediated by integrins activated through the action of inflammatory chemokines such as IL-8 or MIP-1β presented at the surface of endothelial cells. Within hours of the initial stimulus, neutrophils begin to enter the tissue and may continue transmigration for many days. The appearance of inflammatory cells in the surrounding tissue marks the beginning of tissue damage. In some inflammatory conditions, tissue damage is caused by direct injury of the vessels and amplified by the subsequent recruitment of neutrophils into the tissue.

The expression of O-glycans reduces cell-cell interactions because of the bulkiness of these adducts. The expression of core 2 O-glycans is regulated by the transcriptional levels of core 2 GlcNAc-T in all of these cases. Antigen-mediated activation of peripheral T- and B-cells is characterised by increased activity of core 2 GlcNAc-T and branched O-glycans on CD43 (leukosialin) (19-20). Leukocyte extravasation, lymphocyte trafficking and other processes involve O-glycan synthesised by core 2 GlcNAc-T. Specifically, core 2 GlcNAc-T synthesises cell-surface O-glycan structures terminating in sialyl Lewis^x, which is involved in the recruitment of leukocytes to the site of inflammation. Core 2 GlcNAc-T is not important for T-cell development, but overexpression of this enzyme has been shown to completely block the development of myeloid lineages. Overexpression of core 2 O-glycans has also been reported to affect the interaction between T-cells and B-cells (T-B interaction). This T-B interaction is crucial for humoral immune response and is mediated through binding of the CD40 ligand (CD40L) on T-cells with CD40 on B-cells (CD40L-CD40 interaction). This interaction induces the proliferation of B-cells. Overexpression of core 2 O-glycans has been shown to cause a significant reduction in CD40L-CD40 interaction (21).

It is possible to effectively block the initial step of leukocyte invasion from taking place, by blocking the synthesis of sialyl Lewis^x on the cell surface of activated leukocytes and thereby halting their interactions with selectins. Therefore, inhibitors of core 2 GlcNAc-T that can normalise the activity of core 2 GlcNAc-T will prove very useful in stopping the onset of inflammation.

Core 2 GlcNAc-T and diabetic cardiomyopathy

Cardiomyopathy is a condition in which the heart muscle is damaged and no longer functions properly. Cardiomyopathy is divided into three categories: dilated, hypertrophic and restrictive. Restrictive cardiomyopathy results when some disease process, for example diabetes, restricts the movement of the heart.

Although the clinical symptoms of diabetic cardiomyopathy have been identified, its pathogenesis is uncertain. The definition of diabetic cardiomyopathy describes both specific defects in the diabetic's myocytes, such as fibrosis leading to myocardial hypertrophy and diastolic dysfunction, and associated changes in the heart which have developed during the course of diabetes.

There is now strong evidence suggesting that raised activity of core 2 GlcNAc-T is directly responsible for elevated glycoconjugates, commonly observed in the heart tissue of

diabetic animals and patients. In support of this, it has recently been shown that increased core 2 GlcNAc-T activity causes pathology similar to that observed in the heart of diabetic patients after years with the condition, in the heart of diabetic experimental animal models. Studies were carried out using a transgenic mouse with core 2 GlcNAc-T expression driven by a cardiac myosin promoter. At 4 months, a marked hypertrophy of the left ventricle and general hypertrophy of the heart was observed (16-17). These observations suggest that direct inhibition of core 2 GlcNAc-T could prevent or treat cardiomyopathy associated with diabetes and hyperglycaemia.

Core 2 GlcNAc-T and cancer

Altered glycosylation of cell surface glycoproteins has been observed for tumour cells and is involved in the metastatic processes. The core 2 branched structure has been associated with the sialyl Lewis^x (sLe^x) determinant (22-27), and NeuAcα2-3Galβ1-4(Fucα1-3)GlcNAc-R recognised by the monoclonal antibody CSLEX-1 (28). Sialyl Lewis^x acts as a ligand for binding of tumour cells (29-30) and leukocytes (31-32) to selectins (e.g. P- and E-selectin) on the surface of endothelial cells. This interaction with endothelial cells may play an important role during metastatic processes (28). Marked changes in core 2 branching and core 2 GlcNAc-T activities are associated with malignant transformation, leukaemia and carcinomas (21, 33-36). Rat fibroblasts and mammary carcinoma cells transfected with T24H-ras express core 2 O-glycans as they become metastatic tumours (33).

Highly metastatic colonic carcinoma cells express both more sialyl Lewis^x than their low metastatic counterparts and adhere more strongly to E-selectin than poorly metastatic cells. There is a strong correlation between the expression of sialyl Lewis^x in tumour cells and tumour progression (34). Moreover, a good correlation exists between the expression of sialyl Lewis^x in core 2 O-glycans and lymphatic and venous invasion. Since sialyl Lewis^x is recognised by E- and P-selectin and to some extent by L-selectin, these results strongly suggest that interaction between selectin (or selectin-related molecules) and carbohydrates plays a role in tumour progression and metastasis. Recent findings suggest that core 2 GlcNAc-T in combination with α1,3-Fuc-T contributes to the selectin-mediated metastasis in oral cavity carcinomas (35). Moreover, Western blot analysis revealed the presence of a major approximately 150kDa glycoprotein that carries O-linked oligosaccharides recognised by anti-sLe^x monoclonal antibody in sLe^x-positive pre-B leukaemia cell lines. This correlation of core 2 GlcNAc-T with CD15s expression suggests that core 2 GlcNAc-T is a regulator of the cell surface expression of sialyl Lewis^x in human pre-B lymphoid cells. These results indicate that core 2 GlcNAc-T mRNA detected by *in situ* hybridisation reflects the malignant potentials of pulmonary adenocarcinoma, because lymph node metastasis is the most affecting factor to the patient's prognosis.

Expression of sialyl Lewis^x in mouse melanoma B16-F1 by transfection with the enzyme 1,3-fucosyltransferase have also confirmed the importance of sialyl Lewis^x in tumour metastasis. Intravenous injection of the transfected cells into mice formed a large number of lung tumour nodules, while the parent B16-F1 cells scarcely formed tumours. Surprisingly, the transfected B16-F1 cells expressing the highest amount of sialyl Lewis^x did not produce tumours in wild-type mice, but produced more tumours in NK cell-deficient mice than those expressing a moderate amount of sialyl Lewis^x. These results suggest that tumour cells expressing excessive amounts of sialyl Lewis^x are targeted by

NK cells, possibly because C-type selectin receptors on NK cells recognise those tumour cells. These results provide future directions aiming at inhibition of tumour metastasis by manipulating sialyl Lewis^x expression in tumour cells. The expression of sialyl Le^a, sialyl Lewis^x (E-selectin ligand carbohydrate structures) and raised activity of core 2 GlcNAc-T are all closely associated with malignancy of colorectal cancer (36). Recently, Numahata (37) demonstrated that sialyl Lewis^x expression in primary bladder carcinoma is a predictor of invasive and metastatic outcome. No other carbohydrate epitope examined to date has equal prognostic value. Therefore, inhibitors of core 2 GlcNAc-T could prevent or treat cancer.

Core 2 GlcNAc-T and diabetic retinopathy

Diabetes is a disorder characterised by hyperglycaemia or elevated blood glucose. Diabetes is divided into two subgroups: Type 1 diabetes caused by insulin deficiency and Type 2 diabetes caused by insulin resistance. Type 1 and Type 2 diabetes can [illegible] serious complications in virtually every organ system. Chronic complications may [illegible] 10 years to become evident and are [illegible] predominantly by sustained high blood glucose. These long-term complications include microvascular damage to the kidneys (nephropathy), eyes (retinopathy) and nerves.

Diabetic retinopathy is a progressive vision threatening complication of diabetes (38) characterised by capillary occlusion, formation of microvascular lesions and retinal neovascularisation adjacent to ischaemic areas of the retina (39-40).

It has recently been found that raised activity of core 2 GlcNAc-T is directly responsible for increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy (41). It has now also been demonstrated that elevated glucose and diabetic serum increases the activity of core 2 GlcNAc-T and adhesion of human leukocytes to endothelial cells through diabetes activated serine/threonine protein kinase Cβ2 (PKCβ2)-dependent phosphorylation (42-43). This regulatory mechanism involving phosphorylation of core 2 GlcNAc-T is also present in polymorphonuclear leukocytes (PMNs) isolated from Type 1 and Type 2 diabetic patients. Inhibition of PKCβ2 activation with a specific inhibitor, LY379196, attenuates serine phosphorylation of core 2 GlcNAc-T and prevents increased leukocyte-endothelial cell adhesion. PKCβ2-dependent phosphorylation of core 2 GlcNAc-T thus represents a regulatory mechanism for activation of this key enzyme in mediating increased leukocyte-endothelial cell adhesion and capillary occlusion in diabetic retinopathy.

Inhibiting core 2 GlcNAc-T either directly or by inhibiting PKCβ2 can therefore be useful in preventing or treating retinopathy associated with diabetes or hyperglycaemia by inhibiting (to normal basal levels) the activity of core 2 GlcNAc-T.

Fenugreek

Fenugreek is a herb native to south-eastern Europe, northern Africa and western Asia, but is widely cultivated in other parts of the world. Its botanical name is *Trigonella foenum-graecum*.. The seed of the fenugreek plant contains many active compounds with pharmaceutical applications, including iron, vitamin A, vitamin B_1, vitamin C, phosphates,

flavonoids, saponins, trigonelline and other alkaloids. The seeds are also high in fiber and protein.

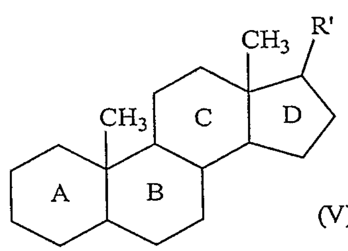
Fenugreek, which contains many active ingredients, such as coumarins, saponins and glycosides, has been used for thousands of years for the treatment of diabetes. Many studies (44) have demonstrated the hypoglycaemic properties of fenugreek in both animals and humans. The hypoglycaemic properties have been attributed to the amino acid 4-hydroxyisoleucine which has potent insulinotropic activity (45-46).

To date, there is no evidence suggesting that an extract prepared from fenugreek seeds can lower blood glucose in Type 1 or Type 2 diabetic patients or experimental animals with diabetes.

Brief Description of the Invention

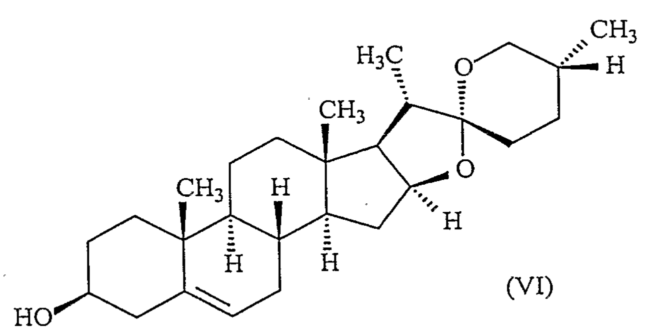
The present invention provides a composition obtainable from fenugreek seeds and comprising a core 2 GlcNAc-T inhibitor. Preferably, the composition is obtained by extraction from fenugreek seeds.

Preferably, the core 2 GlcNAc-T inhibitor of the composition comprises a steroid core. The term "steroid core" means the tetracyclic ring system shown as formula (V)



(V)

wherein R' designates a side chain. The four rings are conventionally designated A, B, C and D. Preferably, the steroid core is diosgenin in open-chain or ring-closed form. Diosgenin itself is in ring-closed form and is of the formula (VI):



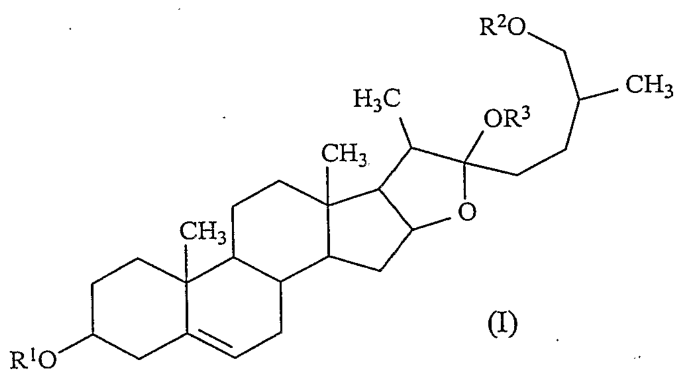
(VI)

The steroid core may comprise a sugar-derived substituent. The term sugar-derived substituent means a saccharide, in which optionally one or more hydrogens and/or one or more hydroxyl groups have been replaced by -R, -OR, -SR, $-NR_2$, wherein R is hydrogen,

methyl, ethyl or propyl. Saccharides include, but are not limited to, monosaccharides, disaccharides, trisaccharides, tetrasaccharides and polysaccharides. Monosaccharides include, but are not limited to, glucose, fructose, mannose, gulose, idose, galatose, talose, allose, altrose, ribose, arabinose, xylose, lyxose, rhamnose, fucose, lyxose and sorbose. Disaccharides include, but are not limited to, cellobiose, lactose, maltose, melibiose, palatinose, sucrose and trehalose. Trisaccharides include, but are not limited to, melezitose and raffinose. Tetrasaccharides include, but are not limited to, stachyose.

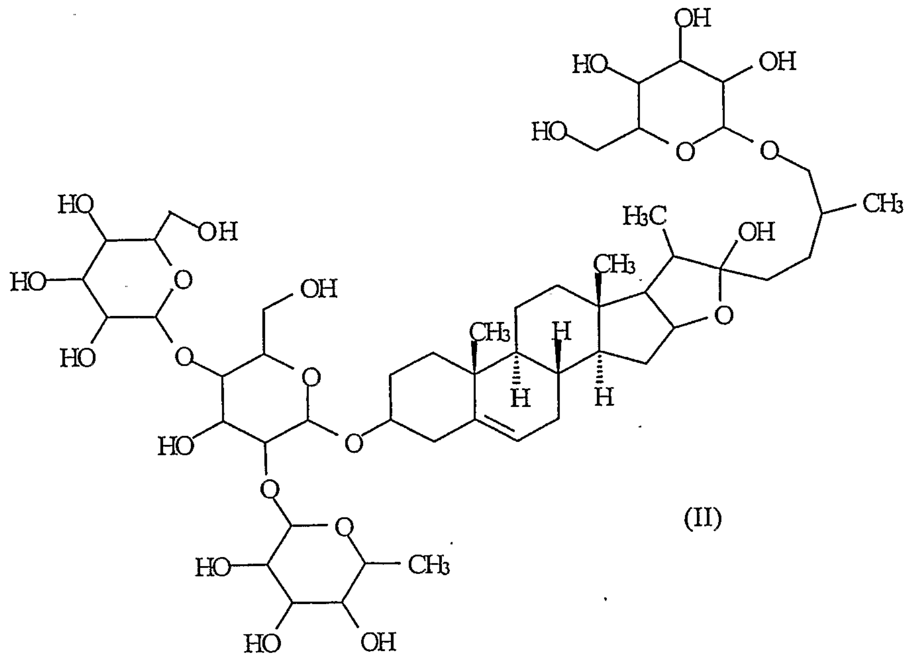
Preferably, the steroid core comprises at least one sugar-derived substituent; more preferably, the steroid core comprises at least two sugar-derived substituents. Preferably, each sugar-derived substituent is independently a mono-, di-, tri- or tetrasaccharide; more preferably, each sugar-derived substituent is independently a mono- or trisaccharide.

Preferably, the diosgenin is in open-chain form and the core 2 GlcNAc-T inhibitor is of the formula (I)



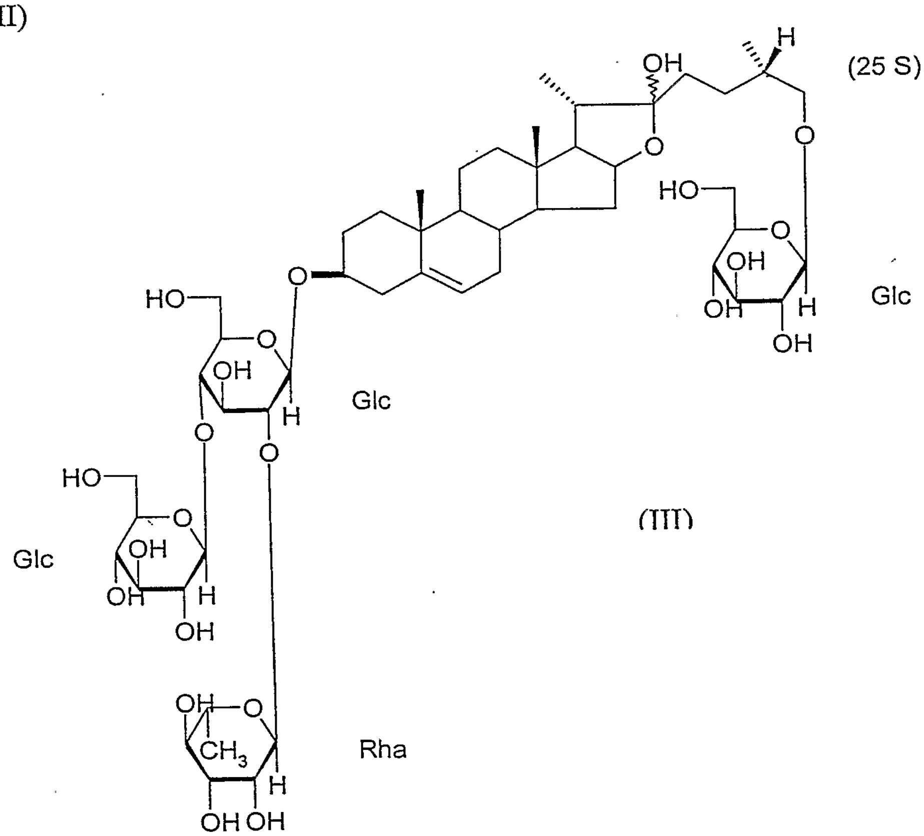
(I) ,

wherein R^1 is a mono-, di-, tri- or tetrasaccharide, R^2 is a mono-, di-, tri- or tetrasaccharide, and R^3 is hydrogen, methyl or ethyl. Preferably, R^1 is a trisaccharide, R^2 is a monosaccharide and R^3 is hydrogen. More preferably, R^1 is a trisaccharide comprising two glucoses and one rhamnose, R^2 is glucose and R^3 is hydrogen. Even more preferably, R^1, R^2 and R^3 are such that the core 2 GlcNAc-T inhibitor is of the formula (II)



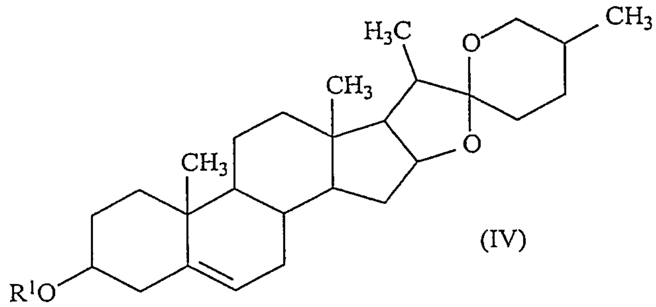
(II)

Most preferably, R^1, R^2 and R^3 are such that the core 2 GlcNAc-T inhibitor is of the formula (III)



(III)

Alternatively, the diosgenin is in ring-closed form and the core 2 GlcNAc-T inhibitor is of the formula (IV)



(IV)

,

wherein R^1 is a mono-, di-, tri- or tetrasaccharide. Preferably, R^1 is a trisaccharide. More preferably, R^1 is a trisaccharide comprising two glucoses and one rhamnose.

The composition may comprise at least 10% of a core 2 GlcNAc-T inhibitor, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90%, and even more preferably at least 95%.

The present invention further provides a method of preparing a composition comprising a core 2 GlcNAc-T inhibitor, the method comprising the steps of:

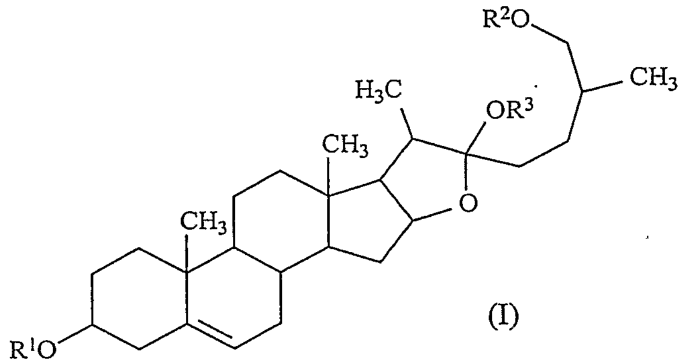
(a) grinding fenugreek seeds to obtain a fenugreek seed powder, and
(b) extracting the fenugreek seed powder with a polar solvent and concentrating the solution to obtain the composition.

The polar solvent used in the extraction step (b) may be an alcohol, a chlorinated hydrocarbon, acetone, ethyl acetate, water or mixtures thereof or supercritical CO_2. Preferably, the polar solvent is methanol, ethanol, dichloromethane or chloroform. Optionally, the polar solvent is ethanol.

Preferably, the method of the present invention further comprises the step of (c) purifying the composition by chromatography to yield a purified composition. The chromatography used in the purification step (c) may be silica-gel flash chromatography or HPLC.

Preferably, the fenugreek seed powder is defatted by washing with a non-polar solvent prior to the extraction step (b). Preferably, the composition is washed with a non-polar solvent prior to the purification step (c). The non-polar solvent may be a hydrocarbon, light petroleum or supercritical CO_2. Preferably, the non-polar solvent is pentane, hexane, heptane, octane or mixtures thereof. Optionally, the non-polar solvent is hexane.

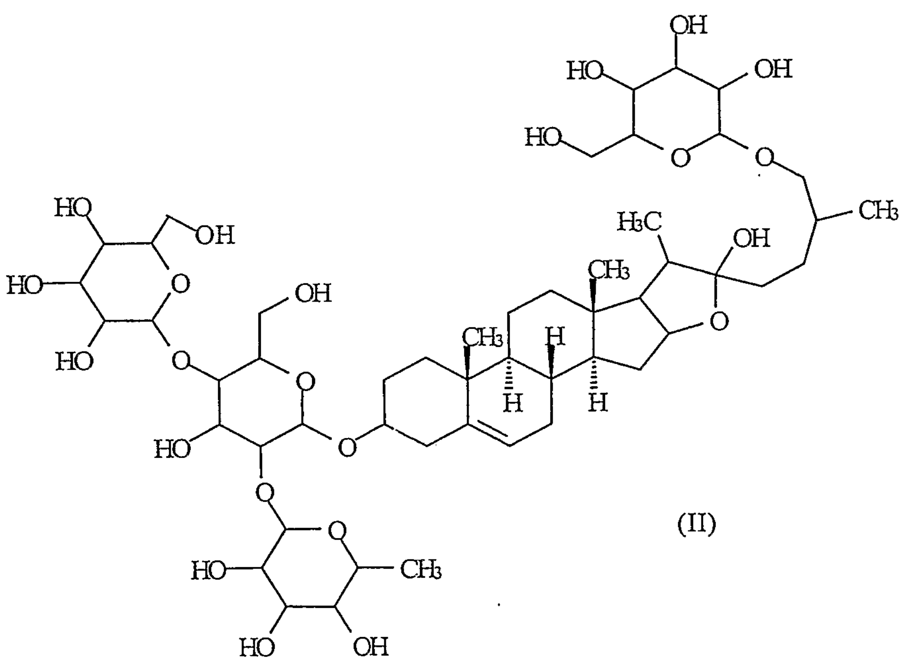
The present invention further provides a compound of the formula (I)



(I)

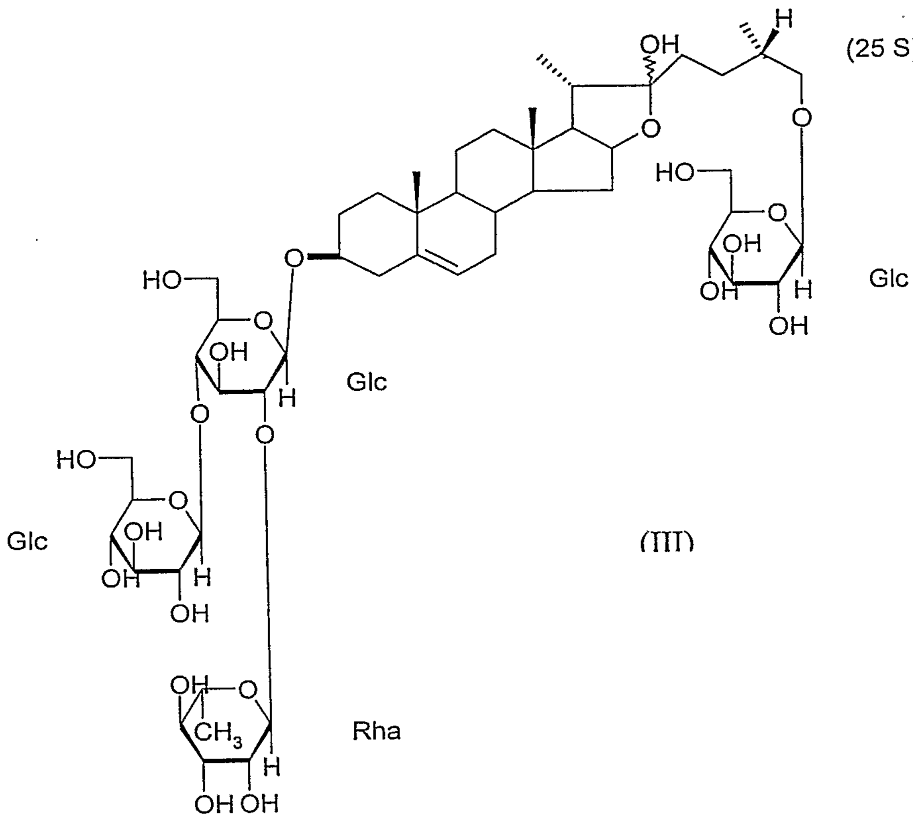
,

wherein R^1 is a mono-, di-, tri- or tetrasaccharide, R^2 is a mono-, di-, tri- or tetrasaccharide, and R^3 is hydrogen, methyl or ethyl. Preferably, R^1 is a trisaccharide, R^2 is a monosaccharide and R^3 is hydrogen. More preferably, R^1 is a trisaccharide comprising two glucoses and one rhamnose, R^2 is glucose and R^3 is hydrogen. Even more preferably, R^1, R^2 and R^3 are such that the compound is of the formula (II)



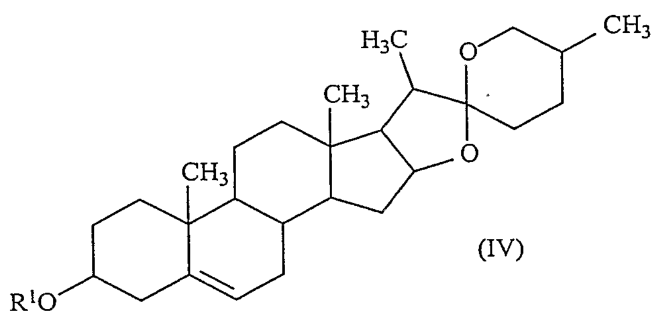
(II)

Most preferably, R^1, R^2 and R^3 are such that the compound is of the formula (III)



(III)

The present invention further provides a compound of the formula (IV)



(IV)

,

wherein R^1 is a mono-, di-, tri- or tetrasaccharide. Preferably, R^1 is a trisaccharide. More preferably, R^1 is a trisaccharide comprising two glucoses and one rhamnose.

The compounds of the present invention can be isolated from fenugreek seeds. Alternatively, they can be synthesised by conventional organic chemistry methods and techniques. Reference in this respect is made to carbohydrate and steroid chemistry books such as "Essentials of Carbohydrate Chemistry and Biochemistry" by Thisbe K. Lindhorst (2000) Wiley, "Carbohydrates in Chemistry and Biology" edited by Beat Ernst, Gerald W. Hart and Pierre Sinay (2000) Wiley, "Essentials of Carbohydrate Chemistry" by John F. Robyt (1998) Springer Verlag, "Carbohydrate Chemistry" by Hassan S. El Khadem (1988), "Carbohydrate Building Blocks" by Mikael Bols (1996), and "Carbohydrate Chemistry" by the Royal Society of Chemistry Staff (1989) CRC Press. Alternatively, the

compounds of the present invention can be prepared by isolation of a precursor from fenugreek seeds and subsequent chemical modification of the precursor.

Preferably, the composition or the compound of the present invention is used as a medicament. More preferably, the medicament is for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T. Even more preferably, the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy. Preferably, the composition or the compound of the present invention is used for lowering the activity of the enzyme core 2 GlcNAc-T.

The present invention further provides fenugreek seeds for use as a medicament for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T. Preferably, the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy. The present invention further provides fenugreek seeds for lowering the activity of the enzyme core 2 GlcNAc-T.

The present invention further provides a pharmaceutical composition comprising the composition, the compound or the fenugreek seeds of the present invention for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T. Preferably, the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

The present invention further provides the use of the composition, the compound or the fenugreek seeds of the present invention for the manufacture of a medicament for lowering the activity of the enzyme core 2 GlcNAc-T.

The present invention also provides the use of the composition, the compound or the fenugreek seeds of the present invention for the manufacture of a medicament for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T. Preferably, the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

The present invention further provides a method of lowering the activity of the enzyme core 2 GlcNAc-T comprising administration of an effective amount of a core 2 GlcNAc-T inhibitor.

Finally, the present invention provides a method of treating a disease associated with raised activity of the enzyme core 2 GlcNAc-T comprising administration of an effective amount of a core 2 GlcNAc-T inhibitor to a patient in need thereof. Preferably, the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

<u>Brief Description of the Drawings</u>

Figure 1 is a schematic flow chart illustrating the biosynthesis of O-glycan core structures.

Figure 2a is a graph illustrating that the activity of the enzyme core 2 GlcNAc-T can be induced by glucose. Human leukocytes (U937) were exposed to normal (5.8 mM) and high glucose (15 mM) for 24 hours at 37°C. Then the cells were lysed and the activity of

core 2 GlcNAc-T measured. The data is presented as the mean ± s.e.m, n = 28, the asterisk representing a significant difference ($P < 0.05$).

Figure 2b is a graph illustrating that crude extract F1 prepared from fenugreek seeds inhibits glucose-induced core 2 GlcNAc-T activity. Human leukocytes (U937) were exposed to normal (N, 5.8 mM; n = 3) and high glucose (G, 15 mM; n = 3) in the presence of fenugreek extract (1:1000 dilution; N-F, G-F). After 24 hours incubation, the activity of core 2 GlcNAc-T was determined in leukocyte cell lysates. The activity of core 2 GlcNAc-T is presented as pmoles/h/mg protein.

Figure 2c is a fluorescence micrograph showing the adhesion of the leukocytes stained with carboxyfluorescein to a monolayer of retinal capillary endothelial cells.

Figure 2d is a graph illustrating that crude extract F1 prepared from fenugreek seeds inhibits adherence of human leukocytes (U937) to cultured retinal capillary endothelial cells. After exposure to elevated glucose (15 mM) the level of leukocyte-endothelial cell adhesion was determined by labelling the leukocytes with carboxyfluorescein. The data is presented as the mean ± s.e.m, n = 3, the asterisk representing a significant difference ($P < 0.05$).

Figure 3 is a graph illustrating that crude extract F1 prepared from fenugreek seeds directly inhibits core 2 GlcNAc-T activity. Human leukocytes (U937) were exposed to 15 mM glucose for 24 hours at 37°C and the activity of core 2 GlcNAc-T was measured in leukocyte cell lysate in the presence of crude fenugreek seed extract (G-F1;1:1000 dilution). The level of core 2 GlcNAc-T activity was measured by determining the formation of core 2 oligosaccharide (attachment of β1,6-linked GlcNAc to the Galβ1,3-GlcNAc-acceptor). The data is presented as mean ± s.e.m of three separate experiments.

Figure 4 is a schematic flow chart illustrating the extraction of fenugreek seeds and the subsequent purification of the fenugreek seed extract.

Figure 5 is a graph illustrating the inhibitory effect of crude fenugreek seed extract F1 and sub-fraction F2 purified from crude extract F1 on glucose-induced activity of core 2 GlcNAc-T in human leukocytes (U937). Cells were exposed to elevated glucose (15 mM) in the presence and absence of sub-fractions F1 and F2. After 24 hours incubation, the core 2 GlcNAc-T activity was determined in leukocyte cell lysates. The data represents the mean of two separate experiments.

Figures 6a and 6b are graphs illustrating the inhibitory effect of sub-fractions F8-F15 purified from crude extract F1 by silica-gel flash chromatography (Biotage) on glucose-induced activity of core 2 GlcNAc-T in human leukocytes (U937). Cells were exposed to elevated glucose (G, 15 mM) in the presence of the sub-fractions. After 24 hours incubation, the core 2 GlcNAc-T activity was determined in leukocyte cell lysates. The data is presented as the mean ± s.e.m, n = 3, the asterisk representing a significant difference ($P < 0.05$).

Figure 7 is a graph illustrating that the aqueous phase of sub-fraction F13 inhibits glucose-induced activity of core 2 GlcNAc-T in human leukocytes (U937). Sub-fractions F9 and F13 were thoroughly mixed with dichloromethane and the aqueous phase was filter-

sterilised and used in the cell-based assay for core 2 GlcNAc-T activity. Human leukocytes were exposed to elevated D-glucose (15 mM) in the presence and absence of the aqueous phases of sub-fractions F9 and F13. The results are presented as the mean of two separate experiments.

Figure 8 is a graph illustrating the inhibitory effect on glucose-induced activity of core 2 GlcNAc-T of sub-fractions purified from the aqueous phase of sub-fraction F13 by HPLC with retention times F18.7-F41.1. Human leukocytes (U937) were exposed to elevated D-glucose (15 mM) in the presence and absence of the HPLC sub-fractions with retention times F18.7-F41.1. The data presented is from one experiment. Sub-fractions G20.24, G20.69, G22.2, G39.9 and G41.1 (represented without a column in Figure 8) were not tested for their inhibitory effect on glucose-induced activity of core 2 GlcNAc-T.

Figure 9 is a graph illustrating the inhibitory effect of HPLC sub-fractions with retention times F19.13 and F19.37. Human leukocytes (U937) were exposed to elevated D-glucose (15 mM) for 24 hours in the presence and absence of the sub-fractions with retention times F19.13 and F19.37 (1: 1000 dilution). The data is presented as the mean ± s.e.m, n =3, the asterisk representing a significant difference ($P < 0.05$).

Figure 10a is a graph illustrating the inhibitory effect on glucose-induced activity of core 2 GlcNAc-T of sub-fractions purified from the aqueous phase of sub-fraction F13 by HPLC with retention times F20.01, F20.29 and F20.55. Human leukocytes (U937) were exposed to elevated D-glucose (15 mM) in the presence and absence of the sub-fractions with retention times F20.01, F20.29 and F20.55 and the activity of core 2 GlcNAc-T was measured after 24 hours. The data is the mean of two separate experiments.

Figure 10b is a chromatogram showing the peaks for sub-fractions F20.01, F20.29 and F20.55 purified from the aqueous phase of sub-fraction F13 by HPLC.

Figure 11 is a graph illustrating that sub-fraction F20.55 directly inhibits core 2 GlcNAc-T in a cell-free assay. After exposing human leukocytes (U937) to 15 mM glucose for 24 hours at 37°C, the cells were lysed and then exposed to heated (H, 100°C) and non-heated (NH) sub-fraction F20.55 (1: 500 dilution). After 30 minutes exposure at 37°C, the activity of core 2 GlcNAc-T was measured. The level of core 2 GlcNAc-T activity was measured by determining the formation of core 2 oligosaccharide (attachment of β1,6-linked GlcNAc to the Galβ1,3-GlcNAc-acceptor). The data is presented as mean ± s.e.m of three separate experiments.

Figure 12 is a schematic illustration of a likely chemical structure of the core 2 GlcNAc-T inhibitor in sub-fraction F20.55 as determined using various analytical techniques.

Figures 13a and 13b are graphs illustrating that elevated glucose increases core 2 GlcNAc-T activity in cultured bovine retinal vascular cells, namely capillary pericytes (Figure 13a) and capillary endothelial cells (Figure 13b). Near confluent cultures were exposed to normal glucose (N, 5.8 mM) and high glucose (G, 15 mM) for 24 hours at 37°C. The cells were lysed and the activity of core GlcNAc-T measured in cell lysates. The data is presented as the mean ± s.e.m (n = 3-4), the asterisk representing a significant difference ($P < 0.05$).

Figures 14a and 14b are graphs illustrating that a crude extract F1 of fenugreek seeds prevents glucose-induced toxicity in cultured bovine retinal vascular cells, namely capillary pericytes (Figure 14a) and capillary endothelial cells (Figure 14b). Cells were exposed to normal (N, 5.8 mM) and high glucose (G, 25 mM) in the presence (N-F, G-F) and absence (N, G) of the fenugreek seed extract. After 4 days incubation, the number of viable cells was determined using a haemocytometer and trypan blue exclusion. The data is presented as the mean ± s.e.m, n = 18 separate experiments, the asterisk representing a significant difference ($P < 0.05$).

Detailed Description of the Invention

Biological activity of crude fenugreek seed extract

As shown in Figure 2a, 24 hour exposure to elevated D-glucose significantly increases the activity of core 2 GlcNAc-T in human leukocytes (U937). It has now been found that crude extract prepared from fenugreek seeds has the potential to inhibit glucose-induced activity of core 2 GlcNAc-T in human leukocytes (Figure 2b) and leukocyte-endothelial cell adhesion (Figure 2d). Leukocyte-endothelial cell adhesion was measured by adding a known number of leukocytes stained with carboxyfluorescein to a monolayer of retinal capillary endothelial cells. The number of attached leukocytes was then counted under a fluorescence microscope using 10-random fields (Figure 2c).

This inhibitory action of the fenugreek seed extract is very similar to that seen with the PKCβ2-inhibitor LY3379196 (Eli Lilly, USA) currently in phase 3 clinical trial for the treatment of proliferative retinopathy and maculopathy. However, while LY3379196 is thought to inhibit core 2 GlcNAc-T by attenuating PKCβ2-dependent phosphorylation, it is now thought that fenugreek seed extract has a direct action on core 2 GlcNAc-T (Figure 3). The results illustrated in Figure 3 were obtained by exposing human leukocytes (U937) to elevated glucose for 24 hours. The cells were then lysed, incubated with crude fenugreek seed extract F1 and core 2 GlcNAc-T activity was measured after 30 minutes incubation.

Preparation and purification of fenugreek seed extracts

Fenugreek seed extracts were obtained as follows (see Figure 4). Fenugreek seeds (Indian fenugreek seeds obtained as Methi seeds from FUDCO, 184 Ealing Road, Wembley, Middlesex, UK) were ground in a hammer mill and filtered through nylon mesh. 820g of the dark-yellow powder obtained were defatted by continuous washing with hexane in a soxhlet apparatus for eight hours. Then the plant material was dried and continuously extracted for 8 hours with ethanol. Filtration to remove solid residues and concentration *in vacuo* of the ethanol yielded a semi-solid brown crude extract labelled F1 (65g). Since this appeared to contain residual oil, 50g of the crude extract F1 were shaken with cold hexane (500ml). The hexane soluble material was filtered off and the solvent removed to give F3 (15.4g), while the insoluble residue was collected on the filter paper and dried to give F2 (27g). The unaccounted weight of about 7g may have been due to losses during transfer or residual solvent in the crude extract F1.

Two separation methods were attempted for extract F2.

The first separation method involved size exclusion chromatography. F2 (1.44g) was chromatographed on a Sephadex G25 column (27.5cm x 3.5cm) using methanol:water 30:70 as mobile phase. Eluting aliquots were compared by TLC and similar ones pooled to give four main sub-fractions F4-F7. In general, this method did not result in a good separation of the components of the fenugreek seed extract.

For the second method, normal phase silica-gel flash chromatography was employed using a commercial kit (Biotage). F2 (5g) was adsorbed onto silica-gel (5g) and packed into the sample barrel that was connected by short tubing to the main chromatography column (20cm x 4cm) containing silica-gel KP-Sil. The sample was eluted onto and through the column with a succession of solvents of increasing polarity consisting of varying mixtures of light petroleum (40/60), chloroform, methanol and acetone. Eluting sub-fractions were examined by TLC and similar ones pooled to give seven main eluted sub-fractions F8 to F14 representing compounds of increasing polarity. The very polar components of F2 remained adsorbed onto the silica in the sample barrel. The silica was removed and shaken with 100% methanol, filtered and dried to give a residue labelled F15. Weights and approximate elution solvents for each sub-fraction are set out in Table 1.

Sub-fraction	**Weight**	**Eluent**
F8	0.03g	light petroleum (40/60) 100% to chloroform 100%
F9	0.10g	chloroform:methanol 90:10
F10	0.02g	chloroform:methanol from 90:10 to 80:20
F11	0.03g	chloroform:methanol from 80:20 to 70:30
F12	0.82g	chloroform:methanol from 70:30 to 60:40
F13	1.58g	chloroform:methanol 50:50
F14	0.01g	chloroform:methanol:acetone 30:30:40 to acetone 100%
F15	0.14g	eluted from silica-gel with methanol

Table 1

Separation of sub-fraction F2 into sub-fractions F8-F15 using flash chromatography.

Biological activity of purified fenugreek seed extracts

The potential of these purified sub-fractions to inhibit glucose-induced activity of core 2 GlcNAc-T in leukocytes was examined. Firstly, it was demonstrated that sub-fraction F2 can inhibit glucose-induced core 2 GlcNAc-T activity in leukocytes (Figure 5). Further experiments demonstrated the presence of the inhibitor of core 2 GlcNAc-T in sub-fractions F13 and F14 (Figures 6a and 6b).

Sub-fractions F9 and F13 were then analysed. An aqueous aliquot (0.5 ml) of both sub-fractions F9 and F13 was extracted with 1 ml of dichloromethane. The components in the organic dichloromethane phase were identified by Hewlett-Packard 6890/5973 GCMS operating in the scan mode. The chromatographs of the two dichloromethane phases were overlaid and peaks found in the biologically active sub-fraction F13, that were not present in the non-active sub-fraction F9, were identified through the use of mass spectral libraries (Table 2). Surprisingly, it was found that the organic dichloromethane phase of the biologically active sub-fraction F13 comprises 4-hydroxy-4-methyl-2-pentanone, a severe respiratory, skin and eye irritant.

Component	Match
4-hydroxy-4-methyl-2-pentanone	good match
octadecanoic acid	good match
decanoic acid	good match
dodecanoic acid	good match
tetradecanoic acid	good match
hexadecanoic acid	good match
chloromethyl-4-chlorooctanoate	poor match – likely to be a chlorinated compound with a similar structure
chloromethyl-9-chlorodecanoate	poor match – likely to be a chlorinated compound with a similar structure
10,10-dimethyl acridine	poor match – likely to be a compound with a similar structure

Table 2

Results of analysis of the organic dichloromethane phase of sub-fraction F13 by GCMS.

In order to ascertain whether the core 2 GlcNAc-T inhibitor was also contained in the aqueous phase of the water dichloromethane mixture, the aqueous phase was removed, filter-sterilised by filtration through 0.22μm filter and used in the cell-based assay for core 2 GlcNAc-T activity. Human leukocytes were exposed to elevated D-glucose (15 mM) in the presence and absence of the aqueous phases of sub-fractions F9 and F13. The results are presented in Figure 7 showing the presence of the core 2 GlcNAc-T inhibitor in the aqueous phase of sub-fraction F13.

Therefore the aqueous phase of sub-fraction F13 was purified by HPLC into sub-fractions F18.7-F41.1 coded by their HPLC retention times. The aqueous phase of sub-fraction F13 was directly injected onto the HPLC operating under reversed-phase conditions (Hewlett-Packard 1050/1100 series). Separation was achieved with an octadecyl-bonded column with a methanol/water mobile phase. Components eluted from the column were detected by a UV detector operating at a fixed wavelength of 22 nm. These components were revealed as peaks on the chromatographic trace from the mass spectrometer detector. The sub-fractions thus obtained were concentrated *in vacuo* to dryness, re-dissolved in phosphate buffered saline (PBS) and filter-sterilised. Cell-based assays for core 2 GlcNAc-T activity were carried out and the results suggested the presence of core 2 GlcNAc-T inhibitor in sub-fractions F19.13-F20.03 (see Figures 8 and 9).

Subsequently larger amounts of the aqueous phase of sub-fraction F13 were purified similarly by HPLC operating under reversed-phase conditions on a phenyl-bonded column with a methanol/water mobile phase into sub-fractions with retention times of 20.01, 20.29 and 20.55, which are equivalent to sub-fractions F19.13, F19.37 and F19.44 above. Cell-based assays for core 2 GlcNAc-T activity confirmed the presence of the core 2 GlcNAc-T inhibitor in these sub-fractions F20.01, F20.29 and F20.55 (Figure 10a). Figure 10b is a chromatogram showing the peaks for sub-fractions F20.01, F20.29 and F20.55 purified from the aqueous phase of sub-fraction F13 by HPLC.

The direct inhibition of core 2 GlcNAc-T by HPLC purified sub-fraction F20.55 has been demonstrated using the cell-free assay system (Figure 11). After exposing human leukocytes (U937) to 15 mM glucose for 24 hours at 37°C, the cells were lysed and then

exposed to heated (H, 100°C) and non-heated (NH) sub-fraction F20.55 (1: 500 dilution). After 30 minutes exposure at 37°C, the activity of core 2 GlcNAc-T was measured. As shown in Figure 11, it was found that sub-fraction F20.55 directly inhibits core 2 GlcNAc-T in a cell-free assay. Heating of sub-fraction F20.55 only slightly altered the level of core 2 GlcNAc-T inhibition.

Structural analysis of the core 2 GlcNAc-T inhibitor

The core 2 GlcNAc-T inhibitor in the sub-fraction F20.55 has been partially identified NMR analysis of a sample dissolved in CD_3OD. The following NMR experiments were performed: 1D proton, 2D DQF-COSY (^1H-^1H correlation) [8 hours], 2D edited HSQC (^1H-^{13}C one-bond correlation with multiplicity editing) [22 hours], 2D TOCSY (^1H-^1H relayed correlation) [2 x 8 hours].

The NMR data suggests an organic core moiety and attached sugar component(s). The sugar component(s) represents probably at least 12 carbons and is most probably a disaccharide. The organic core moiety contains at least 27 protonated carbons (the above NMR experiments provide no information on non-protonated carbons). The organic core is nearly fully saturated, with evidence for only a single double bond.

Figure 12 shows a likely chemical structure of the core 2 GlcNAc-T inhibitor in sub-fraction F20.55 as determined using the various analytical techniques.

1D proton NMR analysis of samples of the two other sub-fractions F20.01 and F20.29 dissolved in CD_3OD indicates that the core 2 GlcNAc-T inhibitors present in the two other sub-fractions are structurally similar to the core 2 GlcNAc-T inhibitor present in sub-fraction F20.55, mainly differing in the nature of the attached sugar units.

^1H and ^{13}C NMR data for the core 2 GlcNAc-T inhibitor in sub-fraction F20.55 is presented in Tables 3 and 4.

Sample	Assignment
0.90 singlet	18-H
1.03 doublet J 6.7Hz	27-H
1.06 singlet	19-H
1.33 doublet J 7.1Hz	21-H
1.77 doublet J 6.4Hz	Sugar-Me
2.24 dq J 6.9Hz	20-H
5.29 multiplet	6-H

Table 3
^1H NMR data (sample in deuteriopyridine)

Sample	Assignment
Aglycone portion	
37.5	1
30.1	2
78.0	3
38.9	4
140.7	5
121.8	6
32.3	7
31.6	8
50.3	9
37.2	10
21.1	11
39.9	12
40.7	13
56.5	14
32.5	15
81.1	16
63.8	17
16.4	18
19.4	19
40.7	20
16.4	21
10.6	22
110.6	23
37.1	24
28.3	25
34.4	26
75.3	27
17.4	28
Sugar portion	
100.2	Glc 1'
77.7	2'
76.3	3'
81.9	4'
77.7	5'
62.1	6'
102.0	Rha 1''
72.5	2''
72.7	3''
74.1	4''
69.5	5''
18.6	6''
105.1	Glc 1'''
75.1	2'''
78.4	3'''
71.6	4'''
78.2	5'''
61.6	6'''
105.1	Glc 1''''
75.2	2''''
78.6	3''''
71.6	4''''
78.4	5''''
62.8	6''''

Table 4

^{13}C NMR data (sample in deuteriopyridine)

The core 2 GlcNAc-T inhibitor and diabetic retinopathy

It has been found that elevated glucose levels significantly increase the activity of core 2 GlcNAc-T in cultured bovine retinal vascular cells, namely capillary pericytes (BRP) and capillary endothelial cells (BREC) (Figure 13). Near confluent cultures were exposed to normal glucose (N, 5.8 mM) and high glucose (G, 15 mM) for 24 hours at 37°C. The cells were lysed and the activity of core GlcNAc-T measured in cell lysates.

It has further been demonstrated that fenugreek seed extract has the potential to reverse glucose-induced toxicity (Figure 14) in cultured bovine retinal capillary pericytes (BRP) and endothelial cells (BREC). Cells were exposed to normal (N, 5.8 mM) and high glucose (G, 25 mM) in the presence (N-F, G-F) and absence (N, G) of the fenugreek seed extract. After 4 days incubation, the number of viable cells was determined using a haemocytometer and trypan blue exclusion. It was found that fenugreek seed extract indeed reverses glucose-induced toxicity in cultured bovine retinal capillary pericytes and endothelial cells. However, it is not established yet whether fenugreek seed extract reverses glucose-induced toxicity by normalising the activity of core 2 GlcNAc-T.

This protection of retinal vascular cells fenugreek seed extract is significant, because damage to retinal vascular cells is a hallmark of early diabetic retinopathy. Diabetic retinopathy in humans is mainly a vascular disease, primarily affecting the capillaries (47). The first ultrastructural and microscopic changes reported are retinal capillary basement membrane thickening and pericyte degeneration, both of which compromise the integrity of the capillary wall. Pericyte degeneration leaves lightly stained compartments in the basement membrane sheath called pericyte "ghosts". Damage to both pericytes and endothelial cells leads to the formation of acellular capillaries.

Treatment

Both fenugreek seed extracts in various stages of purification and core 2 GlcNAc-T inhibitors isolated from the extracts or chemically synthesised can be used for the manufacture of a medicament for the treatment or prevention of a disease associated with raised activity of core 2 GlcNAc-T, in particular inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

This medicament can be administered by oral or parenteral routes, including intravenous, intramuscular, intraperitoneal, subcutaneous, transdermal, airway (aerosol), rectal, vaginal and topical (including buccal and sublingual) administration.

For oral administration, the compounds of the invention will generally be provided in the form of tablets or capsules, as a powder or granules, or as an aqueous solution or suspension.

Tablets for oral use may include the active ingredients mixed with pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavouring agents, colouring agents and

preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatine, while the lubricating agent, if present, may be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material, such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

Capsules for oral use include hard gelatine capsules in which the active ingredient is mixed with a solid diluent, and soft gelatine capsules wherein the active ingredients is mixed with water or an oil such as peanut oil, liquid paraffin or olive oil.

Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

For intramuscular, intraperitoneal, subcutaneous and intravenous use, the compounds of the invention will generally be provided in sterile aqueous solutions or suspensions, buffered to an appropriate pH and isotonicity. Suitable aqueous vehicles include Ringer's solution and isotonic sodium chloride. Aqueous suspensions according to the invention may include suspending agents such as cellulose derivatives, sodium alginate, polyvinyl-pyrrolidone and gum tragacanth, and a wetting agent such as lecithin. Suitable preservatives for aqueous suspensions include ethyl and n-propyl p-hydroxybenzoate.

The fenugreek seed extracts and core 2 GlcNAc-T inhibitors of the present invention may also be presented as liposome formulations.

In general a suitable dose will be in the range of 0.01 to 10 mg per kilogram body weight of the recipient per day of the core 2 GlcNAc-T inhibitor, preferably in the range of 0.2 to 1.0 mg per kilogram body weight per day. The desired dose is preferably presented once daily, but may be dosed as two, three, four, five, six or more sub-doses administered at appropriate intervals throughout the day. These sub-doses may be administered in unit dosage forms, for example, containing 10 to 1500 mg, preferably 20 to 1000 mg, and most preferably 50 to 700 mg of active ingredient per unit dosage form.

Experimental methods

Cell culture: Bovine retinal capillary endothelial cells (BREC) and pericytes (BRP) were established from bovine retinas dissected from eyes of freshly slaughtered cattle as described previously (48). Briefly, the isolated retinas were homogenised in serum-free minimal essential medium (MEM, Gibco, Paisley, UK) and filtered through 85 µm nylon mesh. The trapped microvessels were digested with collagenase-dispase (1mg/ml) for 30 minutes (BRP) and 90 minutes (BREC) at 37°C and filtered through a 53 µm nylon mesh. For growth of endothelial cells (BREC), the digested microvessels were plated in gelatine-coated tissue culture flasks and maintained in MEM supplemented with 10% pooled human serum, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. For growth of pericytes (BRP), the microvessels were plated in tissue culture flasks in growth

medium supplemented with 10% fetal calf serum. The cells were used at passage 2-3. The cells were characterised using morphological criteria and by immunostaining with an antibody against factor VIII related antigen and 3G5-pericyte marker.

The human leukocytic cell-line (U937) was cultured in RPMI supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Cell-based assay of core 2 GlcNAc-T activity: To investigate the potential of fenugreek to pharmacologically inhibit core 2 GlcNAc-T, enzyme activity was measured in leukocytes exposed to normal glucose (5.8 mM) and high glucose (15 mM) for 24 hours at 37°C. After incubation, the cells were lysed and frozen at -20°C until used for the measurement of core 2 GlcNAc-T. The activity of core 2 GlcNAc-T in cultured bovine retinal capillary pericytes (BRP) and endothelial cells (BREC) was also measured.

Cell-free assay of core 2 GlcNAc-T activity: Core 2 GlcNAc-T immobilised on sepharose beads were used for this assay. For core 2 GlcNAc-T immunoprecipitation, as well as for Western blots, a polyclonal antibody against core 2 GlcNAc-T was used. Cells were lysed on ice in the following lysis buffer: 20 mM Tris-HCL, pH 7.4/1% Triton X-100/150 mM NaCl/1mM EDTA/1mM EGTA/0.2mM sodium vandate/1mM PMSF/1μg/ml aprotinin/10μg/ml leupeptin. The lysate was incubated at 4°C for 20 minutes with constant agitation and insoluble material removed by centrifugation (14,000g for 5 minutes at 4°C). The clarified lysate was incubated with staphylococcal protein A-Sepharose Cl-4B conjugated primary antibody for 2 hours with constant agitation at 4°C. The immunoprecipitates were washed with Tris buffered saline (10 mM Tris-HCL, pH 7.4/150 mM NaCl) containing 0.5% Triton X-100 and used in the measurement of core 2 GlcNAc-T in the presence and absence of potential inhibitors.

Measurement of core 2 GlcNAc-T activity: To measure core 2 GlcNAc-T activity, leukocytes were washed in PBS, frozen and lysed in 0.9% Triton X-100 at 0°C. The activity of core 2 GlcNAc-T was measured as described previously (41). Briefly, the reaction was performed in a reaction mixture containing 50 mM 2(N-morpholino) ethanesulfonic acid (MES, Sigma, Dorset, UK), pH 7.0, 1 mM UDP-6 [^{3}H]-N-acetylglucosamine (16,000 dpm/nmol, NEN Life Science Products, Hounslow, UK), 0.1 M GlcNAc (Sigma, Dorset, UK), 1 mM Galβ1-3GalNAcα-p-nitrophenol (Sigma, Dorset, UK) as substrate, and 16 μl of cell lysate (100-200 μg protein) for a final volume of 32 μl. After incubating the mixture for 1 hour at 37°C, the reaction was terminated with 1 ml of ice-cold distilled water and processed on a C18 Sep-Pak column (Waters-Millipore, Watford, UK). After washing the column with 20 ml of distilled water, the product was eluted with 5 ml of methanol. The radioactivity of the samples was counted in a liquid scintillation β-counter (LKB-Wallac, London, UK). Endogenous activity of core 2 GlcNAc-T was measured in the absence of the added acceptor. The specific activity was expressed as pmoles/h/mg of cell protein. In each case, the protein concentration was determined with BioRad protein assay (BioRad, Hertfordshire, UK).

Leukocyte-endothelial adhesion assay: Adhesion of leukocytes to endothelial cells was examined by labelling with carboxyfluorescein (Molecular Probe, UK). The assay is well established (41). Briefly, endothelial cells were grown to a confluent state in order to provide an endothelial cell surface for the adhesion of the carboxyfluorescein-labelled leukocytes (U937). After treatment, the leukocytes were centrifuged (14.000g for 1

minute) and washed twice with serum-free RPMI. The cells were then resuspended in 1 ml of serum-free RPMI containing 50 μg/ml carboxyfluorescein. The cells were counted with a haemocytometer and a known number added to the endothelial cells. After 30 minutes incubation at 37°C, non-adherent leukocytes were removed by washing with serum-free RPMI and the dishes fixed in 3.7% formalin in PBS. Attached leukocytes were counted in 10 random high-powered fields (x 100) by fluorescence microscopy. The results were expressed as percentage of adherent leukocytes/field.

Glucose toxicity: BRP and BREC were plated in 3 cm tissue culture dishes and incubated in growth medium for 24 hours at 37°C. Then the cells were incubated in fresh growth medium containing normal glucose (5.8 mM) or elevated glucose (25 mM) in the absence or presence of fenugreek sub-fractions. After 4 days incubation, the number of viable cells was counted using a haemocytometer and trypan blue and the results expressed as percentage of control (5.8 mM glucose). After treatment, some of the cells were stored for measurement of core 2 GlcNAc-T activity.

Sub-fractionation of crude extract of fenugreek seeds: Crude fenugreek seed extract was fractionated into various sub-fractions using Biotage or Sephadex chromatography. Further fractionation of the aqueous phases of some sub-fractions was carried out using HPLC.

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Claims

1. A composition obtainable from fenugreek seeds and comprising a core 2 GlcNAc-T inhibitor.

2. The composition of claim 1, wherein the composition is obtained by extraction from fenugreek seeds.

3. The composition of claim 1 or claim 2, wherein the core 2 GlcNAc-T inhibitor comprises a steroid core.

4. The composition of claim 3, wherein the steroid core is diosgenin in open-chain or ring-closed form.

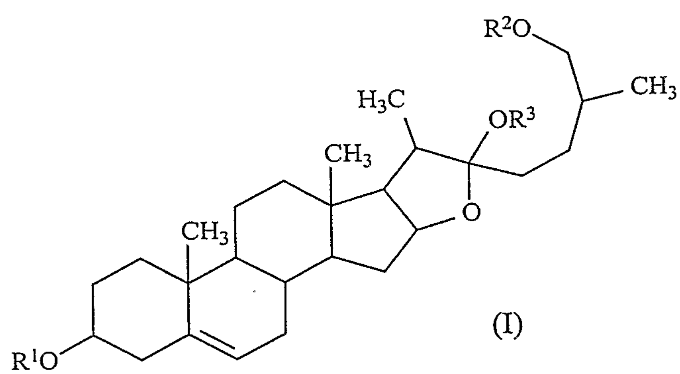
5. The composition of claim 3 or claim 4, wherein the steroid core comprises at least one sugar-derived substituent.

6. The composition of claim 5, wherein the steroid core comprises at least two sugar-derived substituents.

7. The composition of claim 5 or claim 6, wherein each sugar-derived substituent is independently a mono-, di-, tri- or tetrasaccharide.

8. The composition of claim 7, wherein each sugar-derived substituent is independently a mono- or trisaccharide.

9. The composition of claim 4, wherein the diosgenin is in open-chain form and the core 2 GlcNAc-T inhibitor is of the formula (I)



(I)

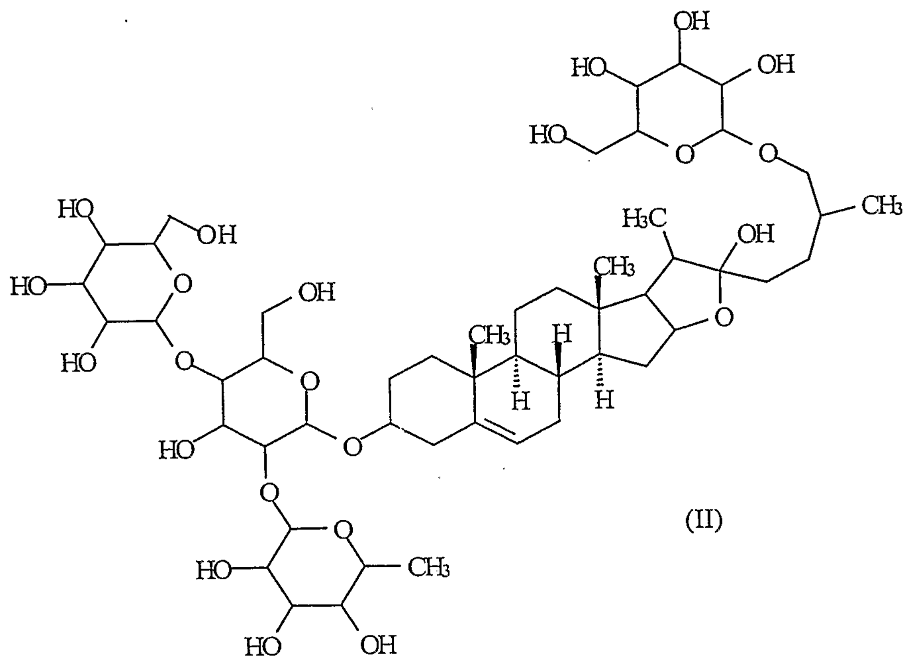
, wherein

R^1 is a mono-, di-, tri- or tetrasaccharide,
R^2 is a mono-, di-, tri- or tetrasaccharide, and
R^3 is hydrogen, methyl or ethyl.

10. The composition of claim 9, wherein R^1 is a trisaccharide, R^2 is a monosaccharide and R^3 is hydrogen.

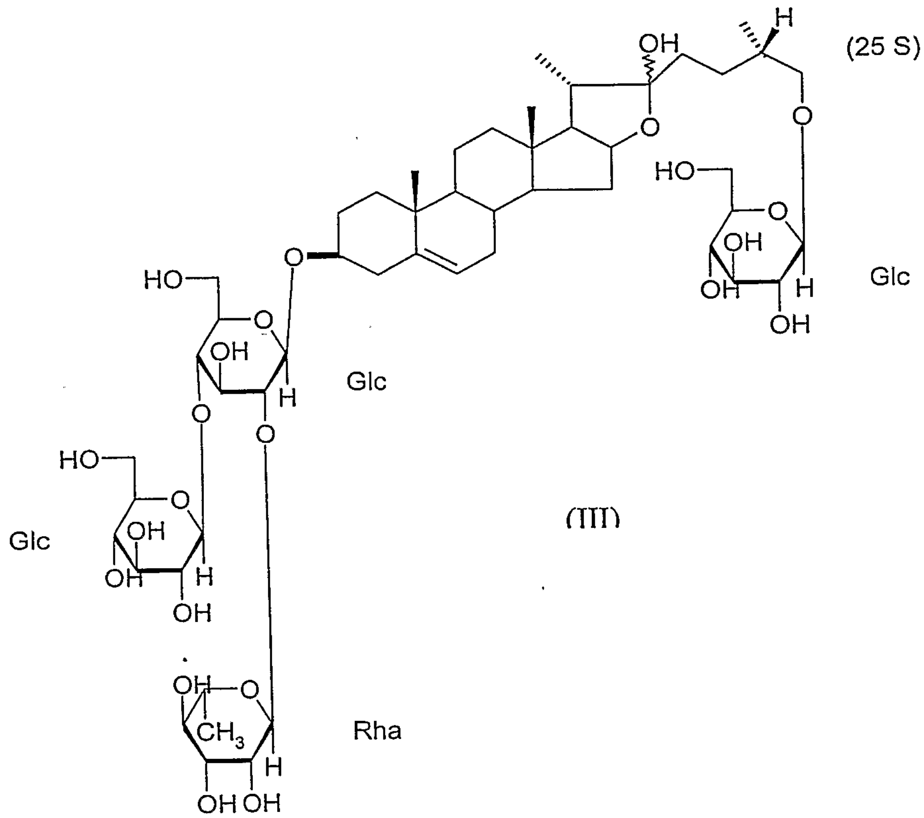
11. The composition of claim 10, wherein R^1 is a trisaccharide comprising two glucoses and one rhamnose, R^2 is glucose and R^3 is hydrogen.

12. The composition of claim 11, wherein the core 2 GlcNAc-T inhibitor is of the formula (II)



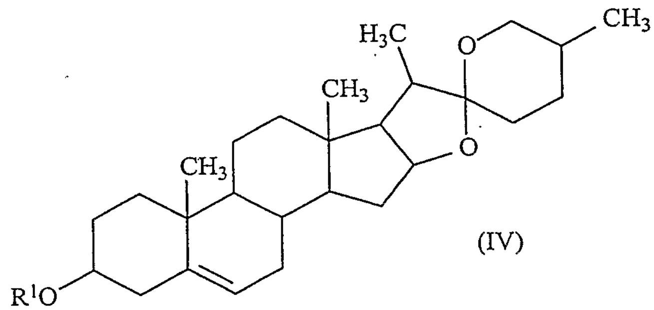
(II)

13. The composition of claim 12, wherein the core 2 GlcNAc-T inhibitor is of the formula (III)



(III)

14. The composition of claim 4, wherein the diosgenin is in ring-closed form and the core 2 GlcNAc-T inhibitor is of the formula (IV)



(IV)

wherein R^1 is a mono-, di-, tri- or tetrasaccharide.

15. The composition of claim 14, wherein R^1 is a trisaccharide.

16. The composition of 15, wherein R^1 is a trisaccharide comprising two glucoses and one rhamnose.

17. The composition of any one of the preceding claims, wherein the composition comprises at least 10% of a core 2 GlcNAc-T inhibitor, preferably at least 20%, preferably at least 30%, preferably at least 40%, preferably at least 50%, preferably

at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 90%, and even more preferably at least 95%.

18. A method of preparing a composition comprising a core 2 GlcNAc-T inhibitor, the method comprising the steps of:
(a) grinding fenugreek seeds to obtain a fenugreek seed powder, and
(b) extracting the fenugreek seed powder with a polar solvent and concentrating the solution to obtain the composition.

19. The method of claim 18, wherein the polar solvent used in the extraction step (b) is an alcohol, a chlorinated hydrocarbon, acetone, ethyl acetate, water or mixtures thereof or supercritical CO_2.

20. The method of claim 19, wherein the polar solvent is methanol, ethanol, dichloromethane or chloroform.

21. The method of claim 20, wherein the polar solvent is ethanol.

22. The method of any one of claims 18 to 21, further comprising the step of:
(c) purifying the composition by chromatography to yield a purified composition.

23. The method of claim 22, wherein the chromatography used in the purification step (c) is silica-gel flash chromatography or HPLC.

24. The method of any one of claims 18 to 23, wherein the fenugreek seed powder is defatted by washing with a non-polar solvent prior to the extraction step (b).

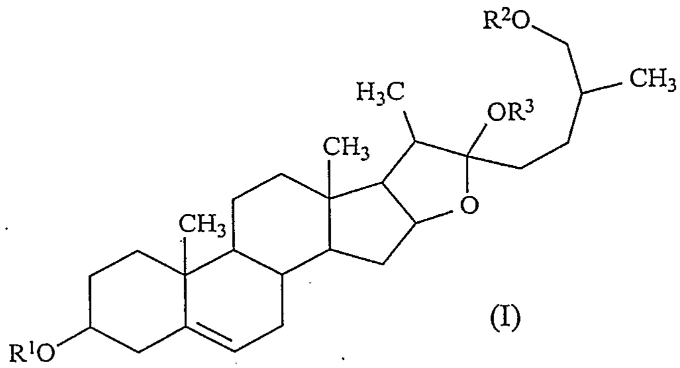
25. The method of any one of claims 18 to 24, wherein the composition is washed with a non-polar solvent prior to the purification step (c).

26. The method of claim 24 or claim 25, wherein the non-polar solvent is a hydrocarbon, light petroleum or supercritical CO_2.

27. The method of claim 26, wherein the non-polar solvent is pentane, hexane, heptane, octane or mixtures thereof.

28. The method of claim 27, wherein the non-polar solvent is hexane.

29. A compound of the formula (I)



(I)

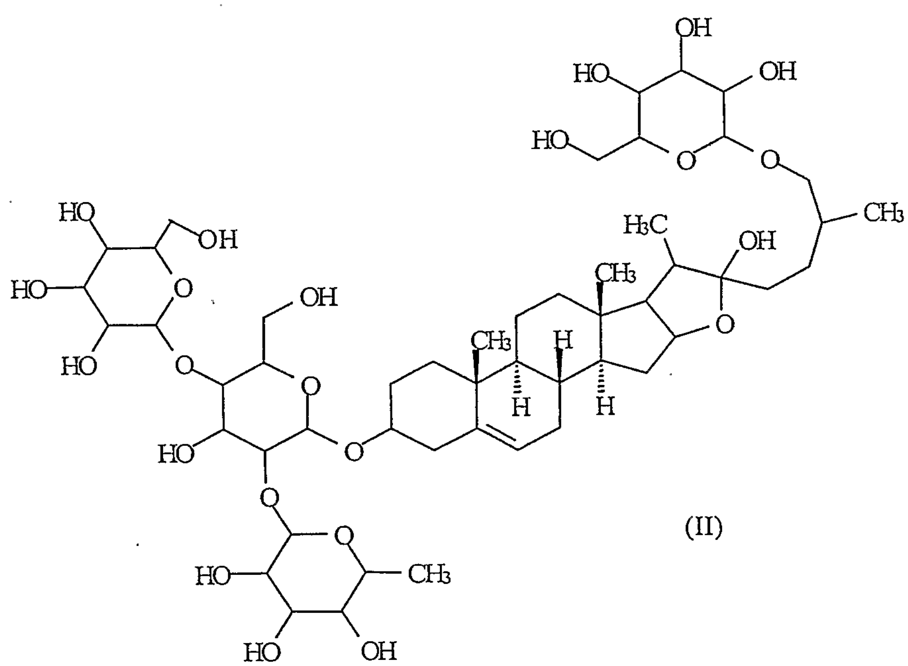
, wherein

R^1 is a mono-, di-, tri- or tetrasaccharide,
R^2 is a mono-, di-, tri- or tetrasaccharide, and
R^3 is hydrogen, methyl or ethyl.

30. The compound claim 29, wherein R^1 is a trisaccharide, R^2 is a monosaccharide and R^3 is hydrogen.

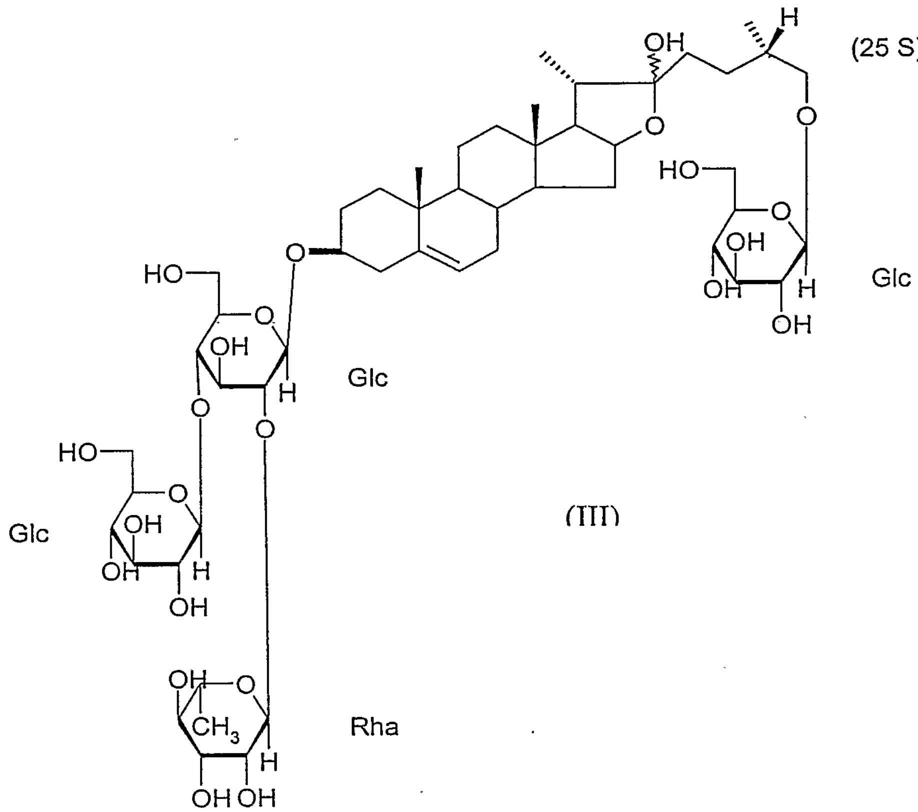
31. The compound of claim 30, wherein R^1 is a trisaccharide comprising two glucoses and one rhamnose, R^2 is glucose and R^3 is hydrogen.

32. The compound claim 31, wherein the compound is of the formula (II)



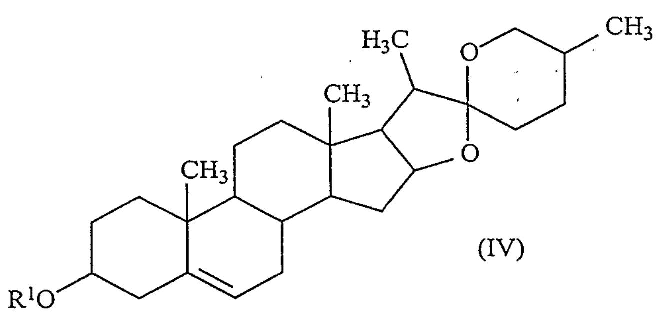
(II)

33. The compound claim 32, wherein the compound is of the formula (III)



(III)

34. A compound of the formula (IV)



(IV)

,

wherein R^1 is a mono-, di-, tri- or tetrasaccharide.

35. The compound of claim 34, wherein R^1 is a trisaccharide.

36. The compound of claim 35, wherein R^1 is a trisaccharide comprising two glucoses and one rhamnose.

37. The composition of any one of claims 1 to 17 or the compound of any one of claims 29 to 36 for use as a medicament.

38. The composition or the compound of claim 37, wherein the medicament is for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T.

39. The composition or the compound of claim 38, wherein the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

40. The composition of any one of claims 1 to 17 or the compound of any one of claims 29 to 36 for lowering the activity of the enzyme core 2 GlcNAc-T.

41. Fenugreek seeds for use as a medicament for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T.

42. The fenugreek seeds of claim 41, wherein the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

43. Fenugreek seeds for lowering the activity of the enzyme core 2 GlcNAc-T.

44. A pharmaceutical composition comprising the composition of any one of claims 1 to 17 or the compound of any one of claims 29 to 36 or the fenugreek seeds of any one of claims 41 to 43 for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T.

45. The pharmaceutical composition of claim 44, wherein the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

46. Use of the composition of any one of claims 1 to 17 or the compound of any one of claims 29 to 36 or the fenugreek seeds of any one of claims 41 to 43 for the manufacture of a medicament for lowering the activity of the enzyme core 2 GlcNAc-T.

47. Use of the composition of any one of claims 1 to 17 or the compound of any one of claims 29 to 36 or the fenugreek seeds of any one of claims 41 to 43 for the manufacture of a medicament for the treatment or prevention of a disease associated with raised activity of the enzyme core 2 GlcNAc-T.

48. The use of claim 47, wherein the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

49. A method of lowering the activity of the enzyme core 2 GlcNAc-T comprising administration of an effective amount of a core 2 GlcNAc-T inhibitor.

50. A method of treating a disease associated with raised activity of the enzyme core 2 GlcNAc-T comprising administration of an effective amount of a core 2 GlcNAc-T inhibitor to a patient in need thereof.

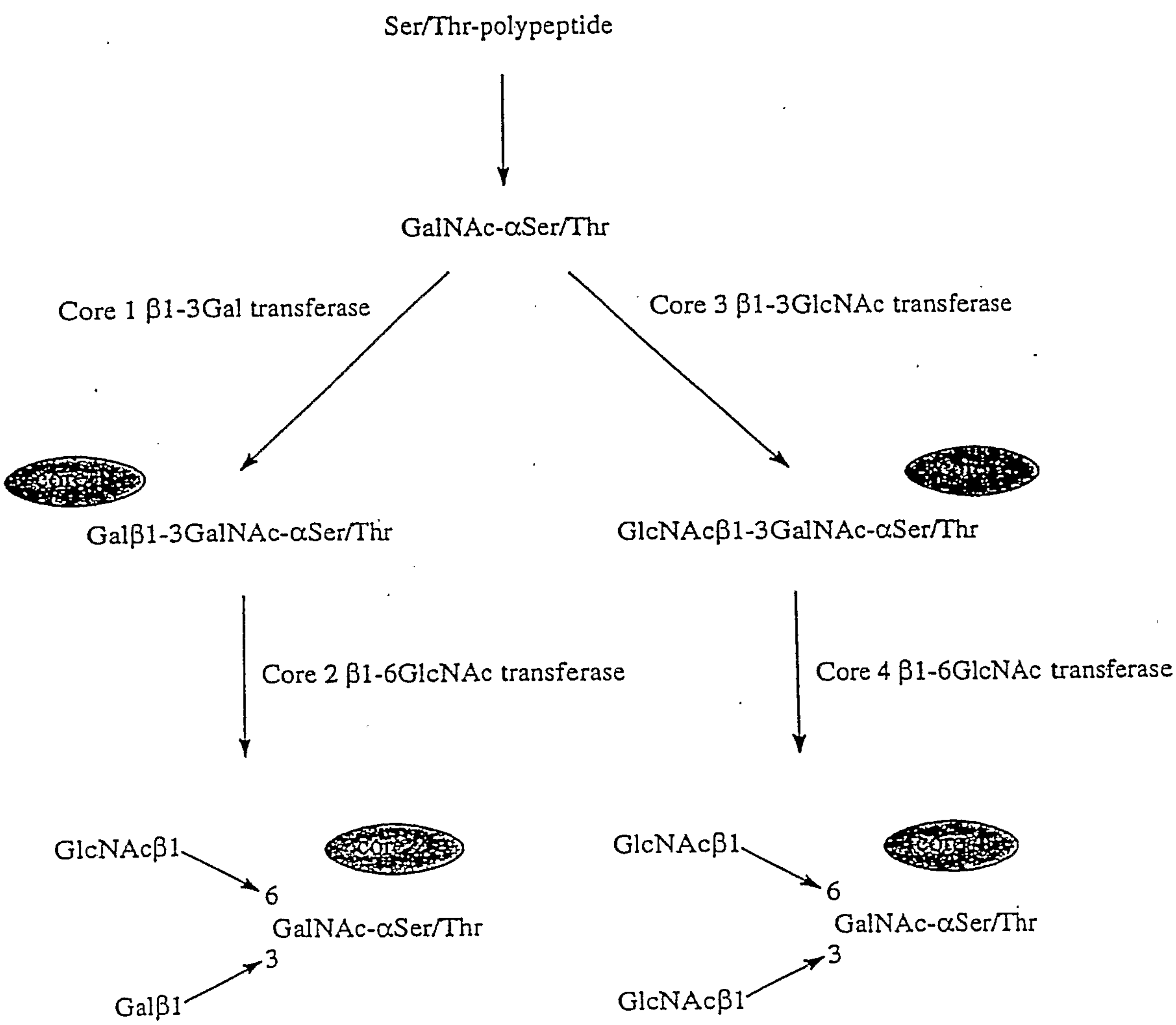
51. The method of claim 50, wherein the disease is an inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

Abstract

Core 2 GlcNAc-T Inhibitor

The present invention relates to a composition obtainable from fenugreek seeds and comprising a core 2 GlcNAc-T inhibitor, a method of preparing the composition and a core 2 GlcNAc-T inhibitor isolatable from fenugreek seeds. The present invention further provides use of the composition or the core 2 GlcNAc-T inhibitor for the manufacture of a medicament for the treatment or prevention of a disease associated with raised activity of core 2 GlcNAc-T, in particular inflammatory disease, diabetic cardiomyopathy, cancer or diabetic retinopathy.

It has been demonstrated in the present invention that a composition prepared from fenugreek seeds can inhibit glucose-induced activity of core 2 GlcNAc-T and glucose-induced binding of human leukocytes to cultured bovine retinal capillary endothelial cells. The administration of the inhibitor to patients can prevent or treat the abnormal formation of core 2 O-glycans and sialyl Lewis^x (sLe^x) by directly inhibiting raised activity of core 2 GlcNAc-T in disease states, such as inflammation, diabetic cardiomyopathy, cancer and diabetic retinopathy.



Ser/Thr-polypeptide
GalNAc-αSer/Thr
Core 1 β1-3Gal transferase
Core 3 β1-3GlcNAc transferase
Galβ1-3GalNAc-αSer/Thr
GlcNAcβ1-3GalNAc-αSer/Thr
Core 2 β1-6GlcNAc transferase
Core 4 β1-6GlcNAc transferase
GlcNAcβ1
6
GalNAc-αSer/Thr
3
Galβ1
GlcNAcβ1
6
GalNAc-αSer/Thr
3
GlcNAcβ1

Figure 1

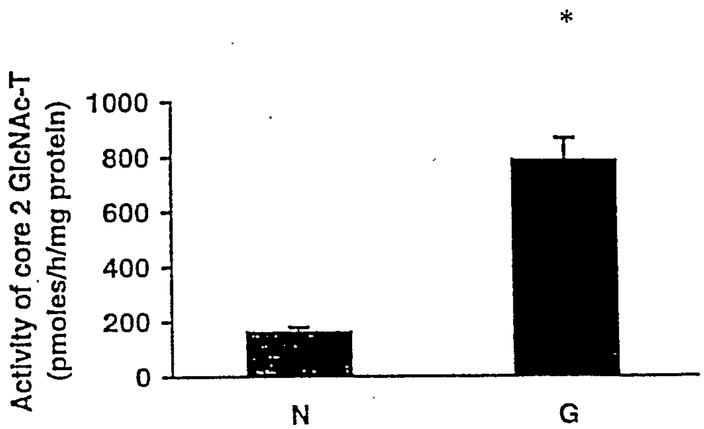


Figure 2a

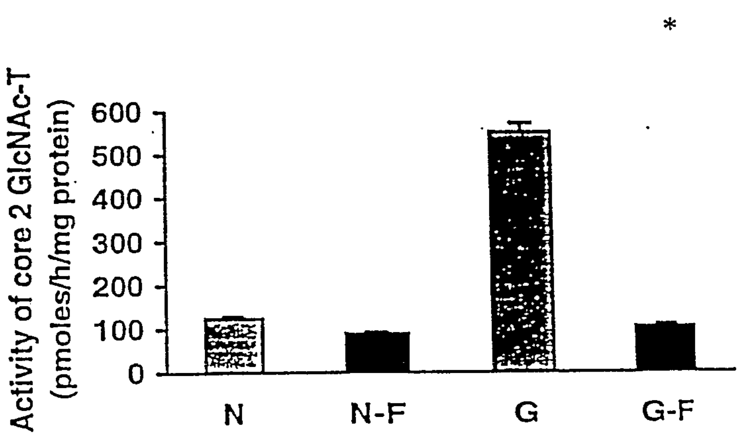


Figure 2b

Figure 2c

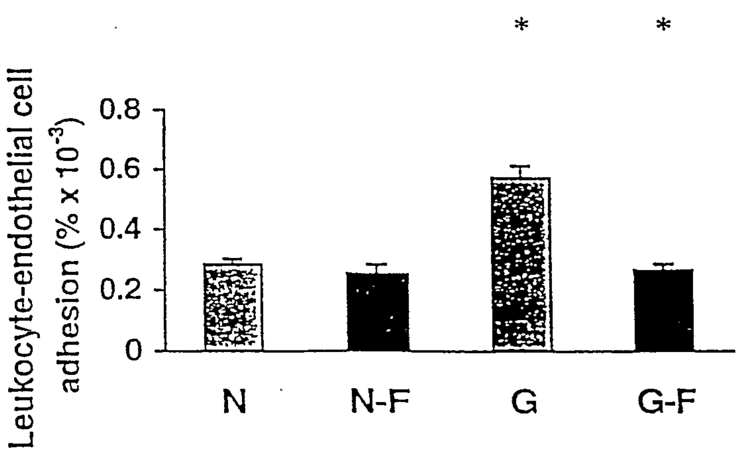


Figure 2d

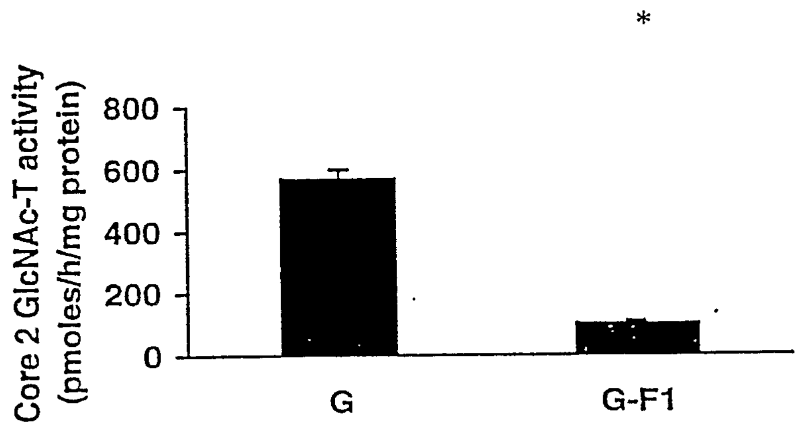


Figure 3

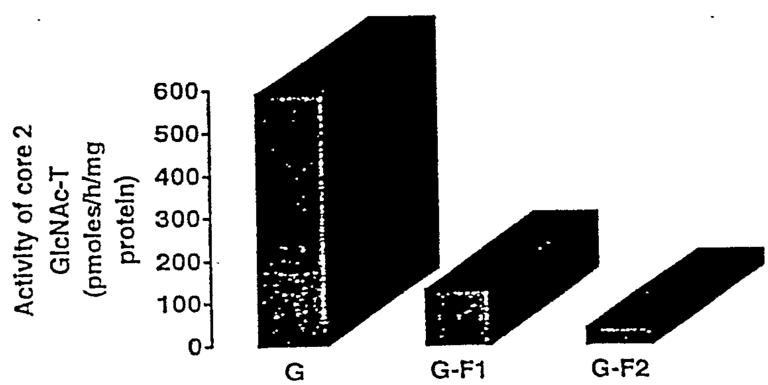


Figure 5

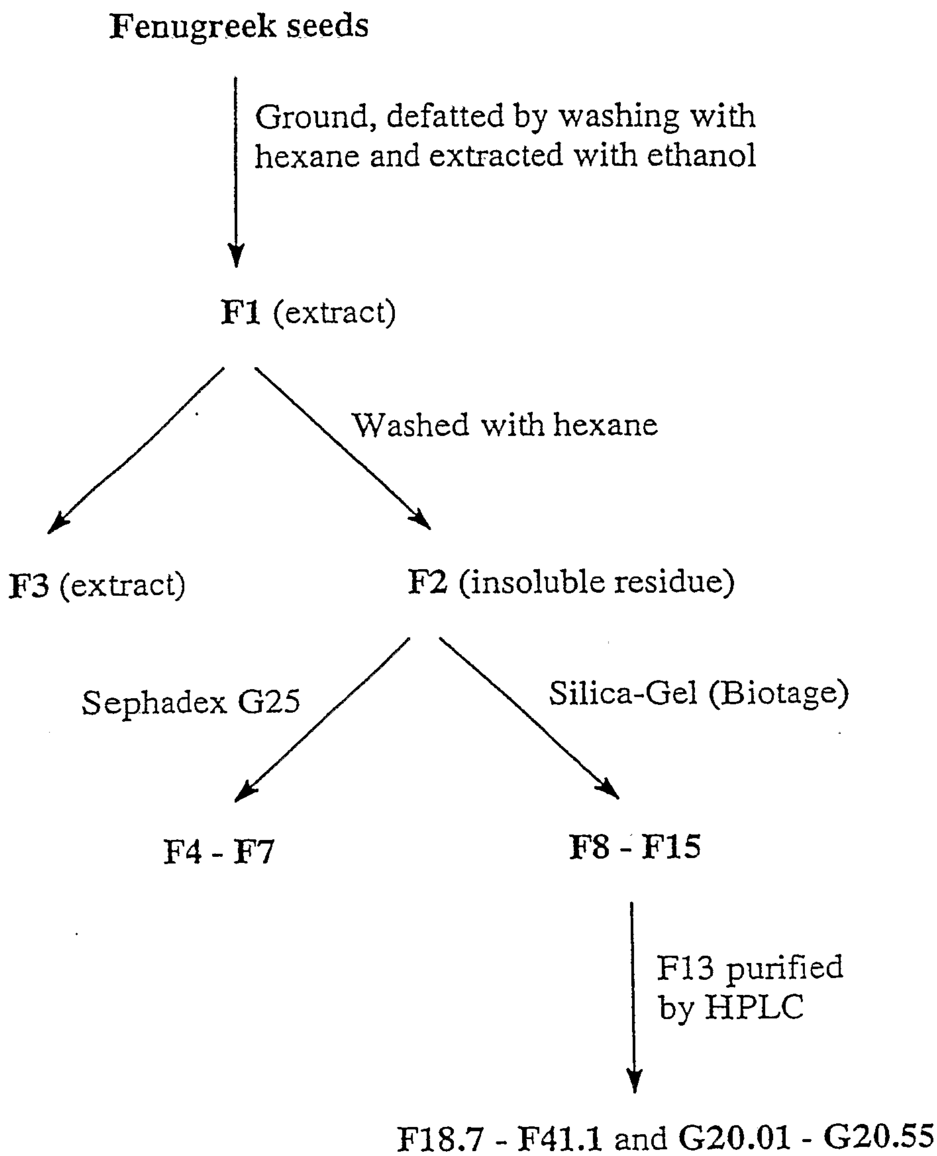

Fenugreek seeds
Ground, defatted by washing with hexane and extracted with ethanol
F1 (extract)
Washed with hexane
F3 (extract)
F2 (insoluble residue)
Sephadex G25
Silica-Gel (Biotage)
F4 - F7
F8 - F15
F13 purified by HPLC
F18.7 - F41.1 and G20.01 - G20.55

Figure 4

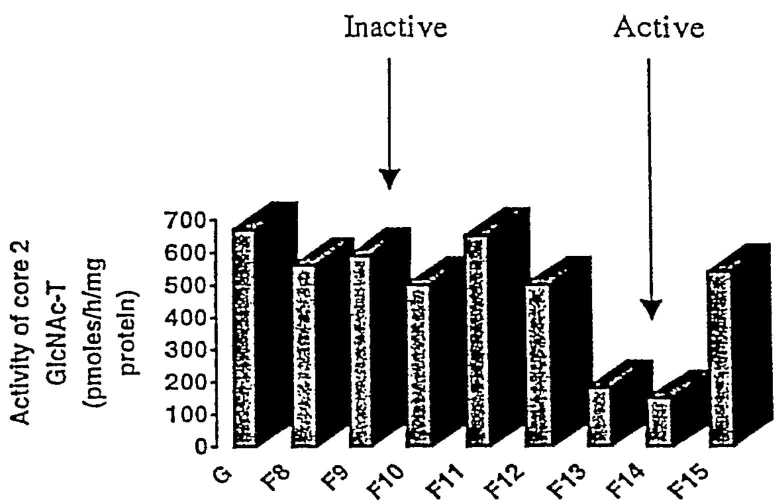


Figure 6a

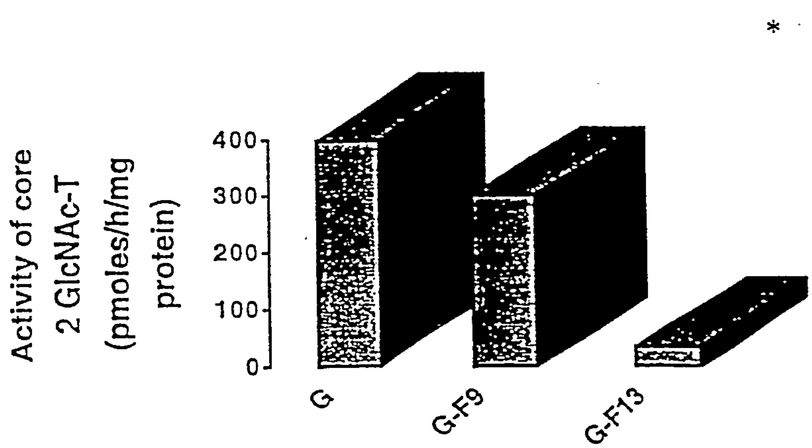


Figure 6b

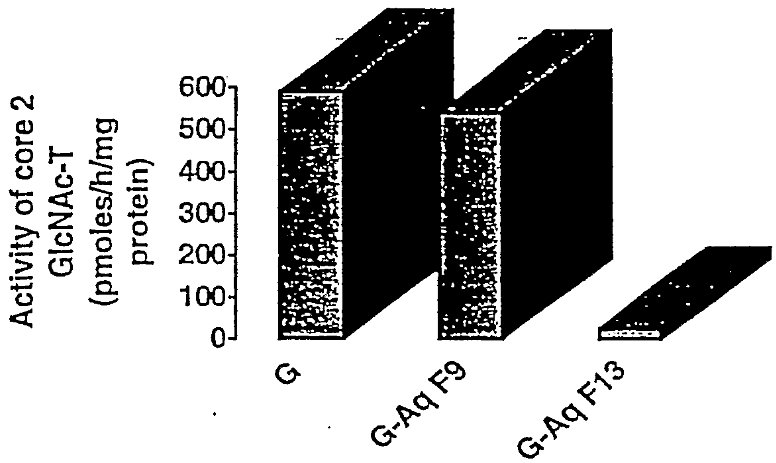


Figure 7

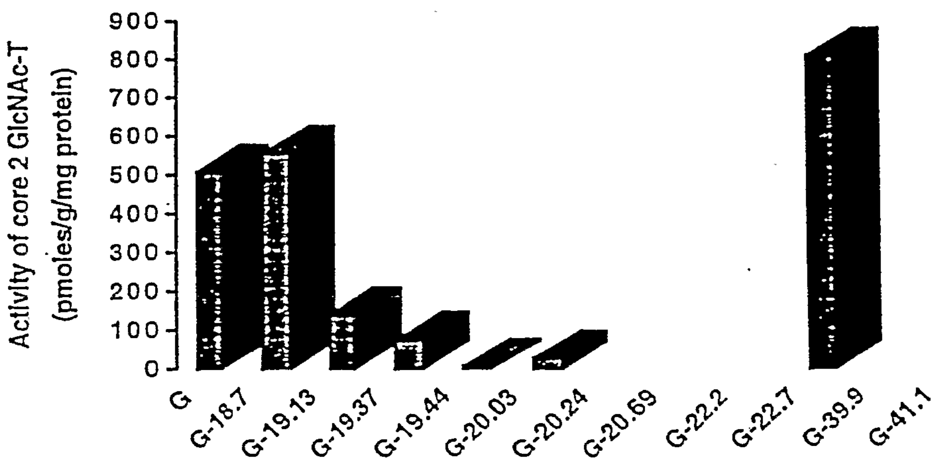


Figure 8

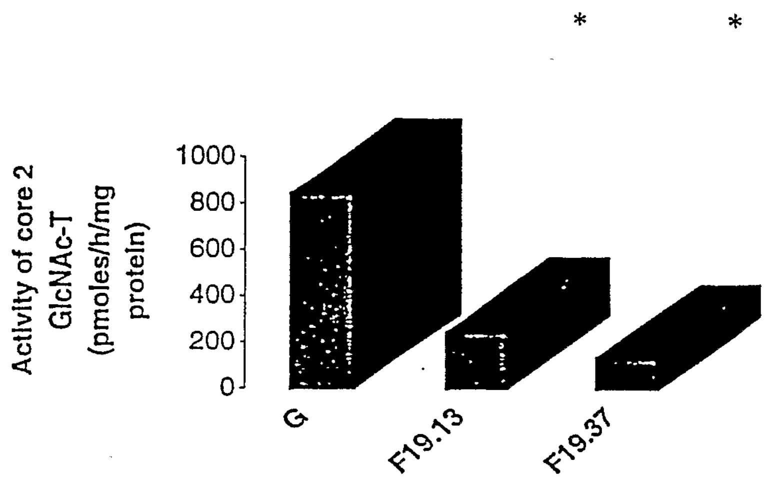

*
*
Activity of core 2 GlcNAc-T (pmoles/h/mg protein)
1000
800
600
400
200
0
G
F19.13
F19.37

Figure 9

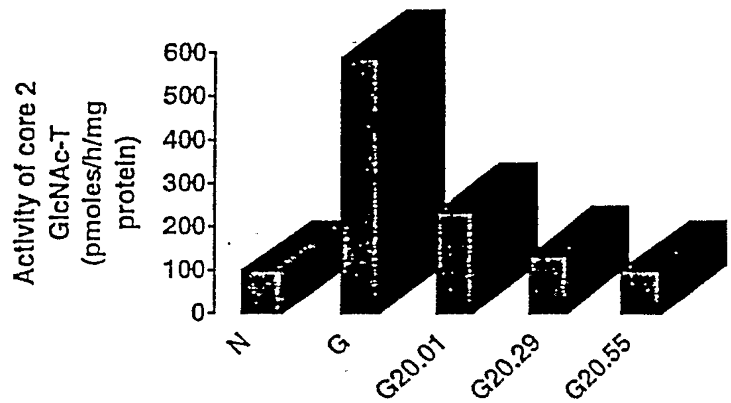


Figure 10a

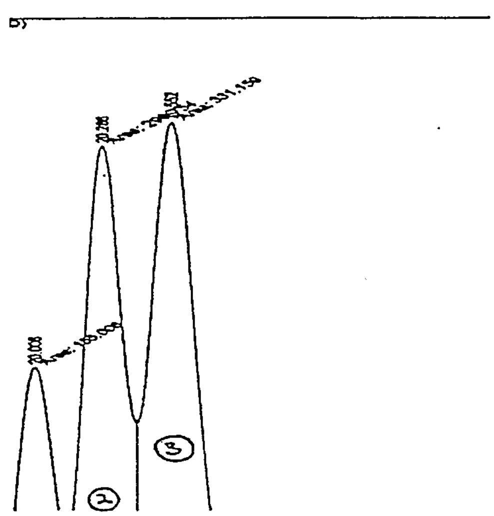


Figure 10b

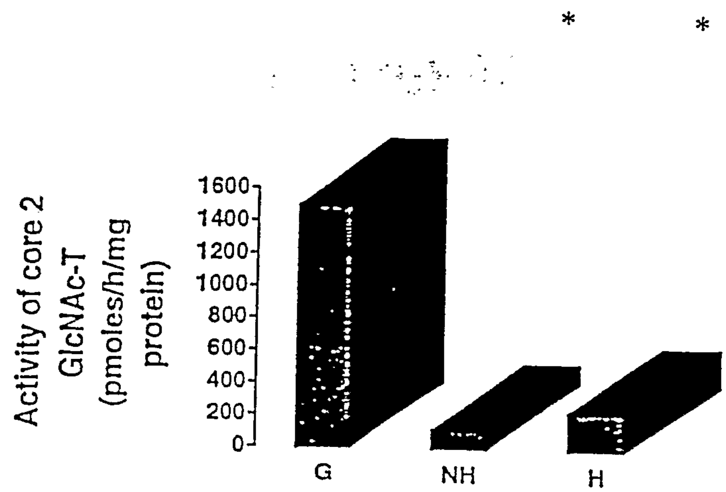
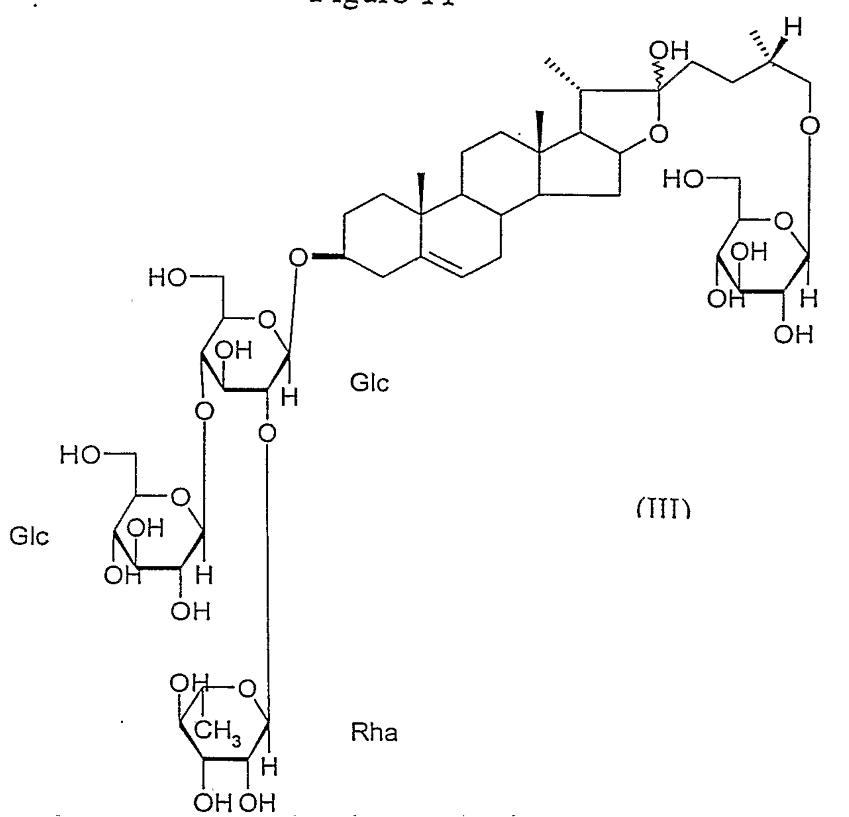


Figure 11



(III)

Figure 12

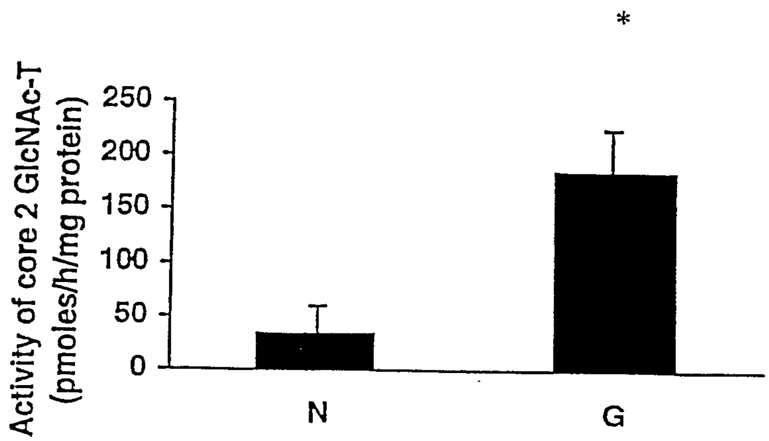


Figure 13a

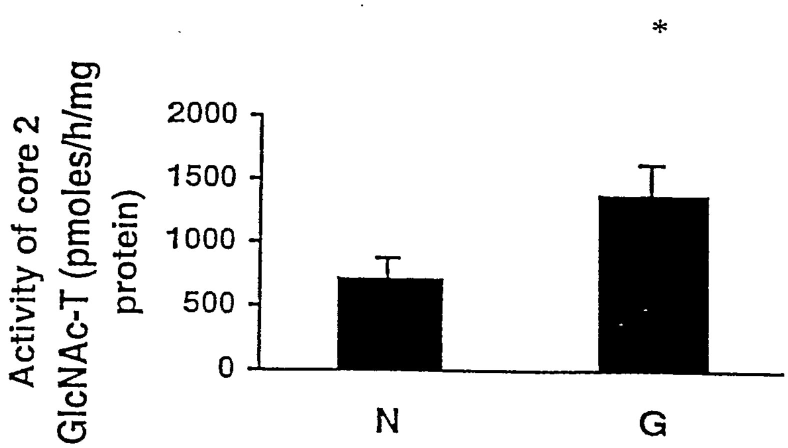


Figure 13b

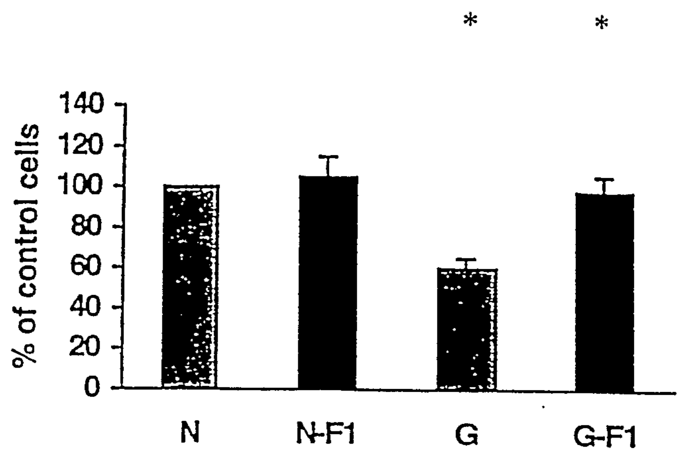


Figure 14a

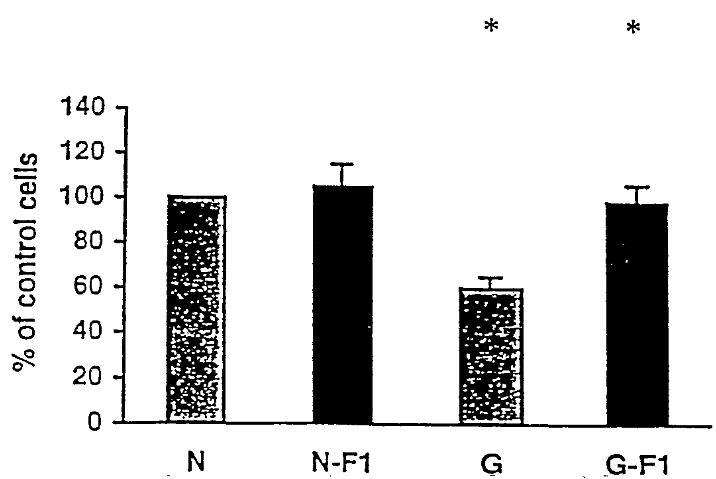


Figure 14b